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A METHOD FOR FIXING FILMS OF HUMAN BLOOD CELLS DURING THE AMEBOID MOVEMENT OF LEUCOCYTES AND THROMBOCYTES.

By M. A. VAN HERWERDEN, M.D.

(*From the Histological and Embryological Laboratory of the University of Utrecht, Utrecht, Holland.*)

PLATE 1.

(Received for publication, January 5, 1920.)

In human blood films fixed in the usual manner, the ameboid movement of leucocytes and thrombocytes is lost. I have therefore endeavored to obtain a simple method to retain this movement in the fixed specimen. Of course, this is possible only by very quick fixation at body temperature, while the cells are in an environment which does not hinder normal motility. The following method gives excellent results (Figs. 1 and 2).

A watch-glass, covered by a second watch-glass, on the inside of which is laid a filter paper moistened with water, is placed in an incubator at a temperature of 38°C. On a well cleaned cover-slip is placed a drop of Deetjen's solution,[1] previously heated to body temperature, to which is added a very small drop of blood from the finger. This cover-slip is placed in the space between the watch-glasses which is kept moist by the wet filter paper. After about 20 minutes in the incubator the covering watch-glass is quickly replaced by another one, on the inside of which a filter paper is placed moistened with a solution of 40 per cent formaldehyde. In this manner the leucocytes and thrombocytes which have continued their ameboid movement, while in the damp space, are quickly fixed at body temperature. After about half an hour the cover-slip is taken out of the incubator and the mixture of Deetjen's solution and blood is carefully run off so that part of the red blood corpuscles, leucocytes, and thrombocytes are left adhering to the cover-slip. The film can now

[1] 0.75 per cent sodium chloride, 0.5 per cent manganese sulfate, 0.01 per cent sodium bicarbonate.

be stained and treated further in the usual way. The best results are obtained if the film is stained while still wet.

In this way well fixed films are obtained. For the thrombocytes, Deetjen's solution is preferable to Ringer's solution, but often good results are also obtained with the latter. The finest ameboid projections of the multinuclear leucocytes as well as those of the mononuclear leucocytes are visible. There is sometimes a radiation of protoplasm around the nucleus of the lymphocytes; the other leucocytes are very irregular in shape. Moreover, the change of shape of the nuclei during the ameboid movement of the cell, seldom visible in the living specimen, can be studied in this way.

Comparison of the size of ameboid leucocytes with that of equally well fixed red blood corpuscles makes it evident that the impression ordinarily obtained of the size of these bodies is most incomplete. This is also true of the thrombocytes. In films made by the method described, the latter generally are of various sizes and shapes. Thrombocytes are found which are as large as red blood corpuscles; others again are much smaller, but also with ameboid projections. The nucleus is scarlet if stained according to Romanowsky's method; it is dark or light blue after being stained in hematoxylin for 24 hours; but it is most distinct if stained with Heidenhain's iron-hematoxylin. By the last method all grades of transition from small pycnotic to large pale nuclei can be observed. In some of the thrombocytes small basophil granules are given off by the nucleus, or the latter is entirely split up into granules. Among the numerous thrombocytes examined I have never been able to distinguish a mitotic division; in some films a constriction and doubling of the nucleus have been seen. In the latter case, as shown in Fig. 1, probably cell division was about to take place.

In films prepared in this way, one can study the position of the mitochondria in the ameboid leucocytes, especially if after being fixed with formaldehyde they have been left for a few days in a solution of 3 per cent potassium bichromate and have then been stained in Heidenhain's iron-hematoxylin (method of Regaud). Sometimes mitochondria and chondriocontes continue into the cell processes.

The method described above demands, beyond careful treatment, so little technical practice that it can very well be carried out by students during practical work.

It may also be interesting from the clinical point of view to observe leucocytes and thrombocytes in their natural shapes, and perhaps to examine the behavior of blood parasites by this method. The method also permits a study of the influence of different substances (poisons, etc.) upon ameboid movement. For instance, it has been noted that an isotonic solution of potassium chloride and sodium chloride, if used instead of Deetjen's solution, allows the ameboid movement to continue (treatment for 1 hour). The same result is obtained with 0.0001 N phenylurethane (in Deetjen's solution), while the same narcotic in a concentration of 0.001 N inhibits motility in less than 1 hour. These are only a few examples of the observations made.

This method has also been used to study the influence of radium radiation on the motility of leucocytes. On the cover-slip that is to carry the drop of Deetjen's solution and the drop of blood is placed a ring of paraffin about 2 mm. in height to support the radium capsule. A control specimen, prepared in like manner, is covered by a second cover-slip, instead of by the radium capsule. The rest of the procedure has been outlined above. The radium capsule used for the experiment contained 3.1 mg. of radium bromide under a mica cover. The blood mixture irradiated with this for 8 hours at body temperature contained several leucocytes which had kept up their ameboid movement. The same intensity of irradiation kills the eggs of *Daphnia pulex* in a much shorter time (generally a few minutes), as demonstrated in a former paper.[2]

In conclusion it may be mentioned that by this method it might be possible to obtain a better view than has hitherto been possible of the morphological changes of thrombocytes during coagulation of the blood.

EXPLANATION OF PLATE 1.

FIG. 1. Cells of human blood fixed during ameboid movement. The film has been stained with Heidenhain's iron-hematoxylin. *l.*, leucocyte; *thr.*, thrombocyte; *r.bl.*, red blood corpuscle. Magnification × 1,000.

FIG. 2. Blood film stained with iron-hematoxylin. At the left one multinuclear leucocyte is shown. The other ameboid bodies are thrombocytes of different sizes.

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[2] Van Herwerden, M. A., *Proc. Koninklÿke Akad. Wetenschappen*, 1918, xxi, 540; *Genetica*, 1919, i, 305.

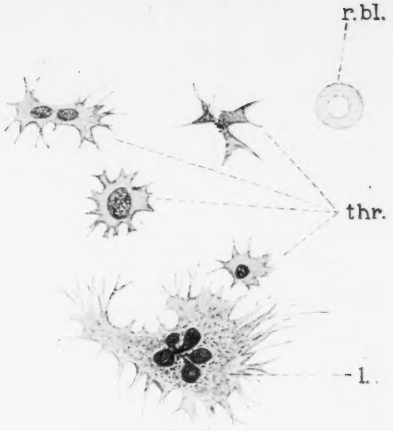


FIG. 1.

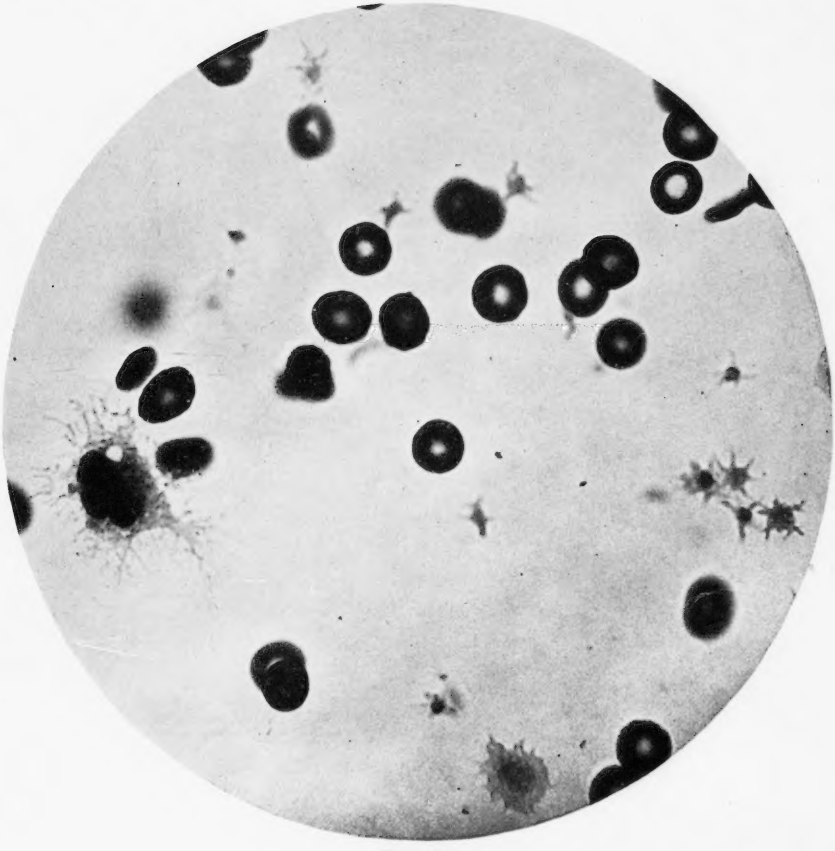


FIG. 2.

(Van Herwerden: Fixing of human blood cells.)

SEGMENTING TERTIAN MALARIAL PARASITES ON RED CORPUSCLES SHOWING LITTLE OR NO LOSS OF HEMOGLOBIN SUBSTANCE. EVIDENCE OF MIGRATION.*

By MARY R. LAWSON, M.D.

(From the Laboratory of Dr. Mary R. Lawson, New London.)

PLATES 2 AND 3.

(Received for publication, April 12, 1920.)

Migration of Malarial Parasites.

The malarial parasite has been described as attaching itself when young to a red corpuscle and remaining there during the entire period of its life cycle, the destruction of the infected corpuscle corresponding to the segmentation of the parasite. But this is not so. Each parasite destroys several red corpuscles, migrating to a fresh corpuscle as soon as one is destroyed. I have seen migrating parasites in eight different stages of development in tertian infections, the oldest form being the presegmenting parasite (Figs. 1 to 5).

Segmenting Parasites Attached to Red Corpuscles Whose Hemoglobin Is Intact or Nearly So (Figs. 6 to 49).

Thayer,[1] discussing tertian parasites, states: "In certain instances, however, sporulating forms may be found within corpuscles which are no larger than the normal red cell, and which are but little decolorized." He writes that Bastianelli and Bignami[2] are inclined to believe that such bodies are more common in cases of anticipating tertian fever. But Thayer states that his observations have not been sufficient to justify

* Aided by a grant from The Rockefeller Institute for Medical Research.

[1] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 55.

[2] Bastianelli and Bignami, quoted by Thayer,[1] p. 55.

the formation of a definite opinion concerning this point. He makes no attempt to explain the occurrence; but he has pictured two segmenting tertian bodies on normal appearing red cells, designating them as instances of "precocious segmentation."[3]

It seems to me that the presence of segmenting parasites on red corpuscles with hemoglobin intact or nearly so is evidence that the parasites have recently attached themselves and have not had sufficient time to cause any appreciable damage to the infected cells and the pigment in connection with these segmenting parasites is additional evidence of their previous attachments to red corpuscles.

Infected Red Corpuscles of Tertian Infections.

One of the most striking features of tertian infections is the rapidity with which the parasites enlarge and decolorize the infected cells. Even the very young parasites do this soon after their attachment, and segmenting parasites are most frequently described as attached to decolorized cells. Marchiafava and Bignami[4] write: "one of the most characteristic properties of the tertian parasite is the rapidity with which the infected red cell becomes decolorized and swollen." The infected corpuscles in the accompanying plates show such a slight amount of alteration due to parasitic action that it is obvious that they could not have been occupied by the attached parasites for the entire period of their development.

While all observers have noted that the infected corpuscle is enlarged as a result of parasitic action, there are also instances in which it is enlarged as a result of the large size of the attached segmenting body. For instance, the segmenting bodies illustrated in Figs. 22 to 25 are larger than normal corpuscles at the same magnification, and they are too large to occupy corpuscles the size of those pictured in Figs. 7 to 10 without enlarging the corpuscles. The red corpuscles being more elastic than the parasites are readily enlarged when pressure is exerted. This mechanical enlargement of a corpuscle means a thinning of its substance, consequently it is usually paler than one

[3] Thayer,[1] Plate 1, Figs. 16 and 17.

[4] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 63.

of normal size. The increased size of many of the infected cells shown in the accompanying plates is the result of the large size of the attached parasites rather than of parasitic action.

Segmenting Bodies of Tertian Infections.

The tertian segmenting bodies are larger than those of the quartan or æstivo-autumnal infections. Within certain limits, from about 14 to 26, they show no fixed number of segments; occasionally I have seen fewer than 14, rarely more than 26. When more than 26 are seen it means, usually, that two segmenting bodies are attached to one corpuscle. It is interesting to note that the small segmenting body does not necessarily mean that the parasite has fewer segments. The pigment in connection with these bodies is usually collected in a mass, but it may be scattered among the spores. The young parasites resulting from the segmentation of these bodies attached to healthy appearing corpuscles frequently attach themselves independently and remain until the corpuscle is destroyed.

SUMMARY.

1. Malarial parasites destroy more than one red corpuscle, migrating to another as soon as one is destroyed.

2. Pigmented segmenting parasites attached to red corpuscles whose hemoglobin is intact or nearly so are *prima facie* evidence of migration.

3. Infected corpuscles in tertian infections may be enlarged as a result of the large size of the attached parasite as well as by parasitic action.

4. The young parasites resulting from the segmenting bodies attached to healthy red corpuscles usually attach themselves independently to the infected corpuscle and remain there until the corpuscle is destroyed.

EXPLANATION OF PLATES.

PLATE 2.

TERTIAN PARASITES.

Magnification × 1,807.

FIGS. 1 to 5. Presegmenting parasites free in the blood serum. These are parasites in the last free stage of migration.

FIG. 6. A presegmenting parasite attached to a red corpuscle which is enlarged but with hemoglobin nearly intact.

FIGS. 7 to 11. Segmenting parasites attached to red corpuscles which are practically normal both in size and hemoglobin content.

FIGS. 12 to 24. Segmenting parasites attached to red corpuscles whose hemoglobin is practically intact; the cells are enlarged slightly owing, in Figs. 22 to 24, to the large size of the attached parasites.

FIG. 25. A presegmenting parasite attached to a red corpuscle which is normal in hemoglobin content but enlarged by the large size of the parasite.

PLATE 3.

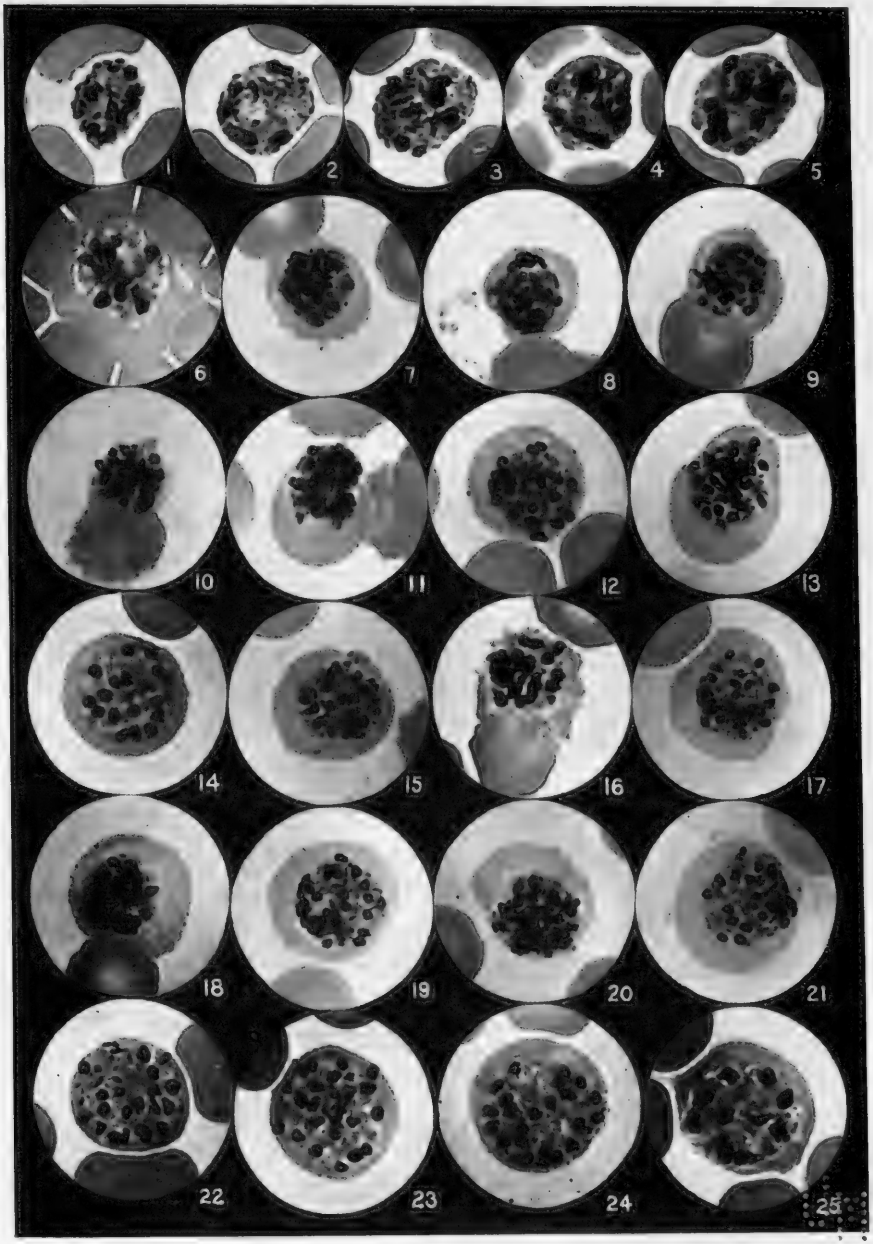
TERTIAN PARASITES.

Magnification × 1,807.

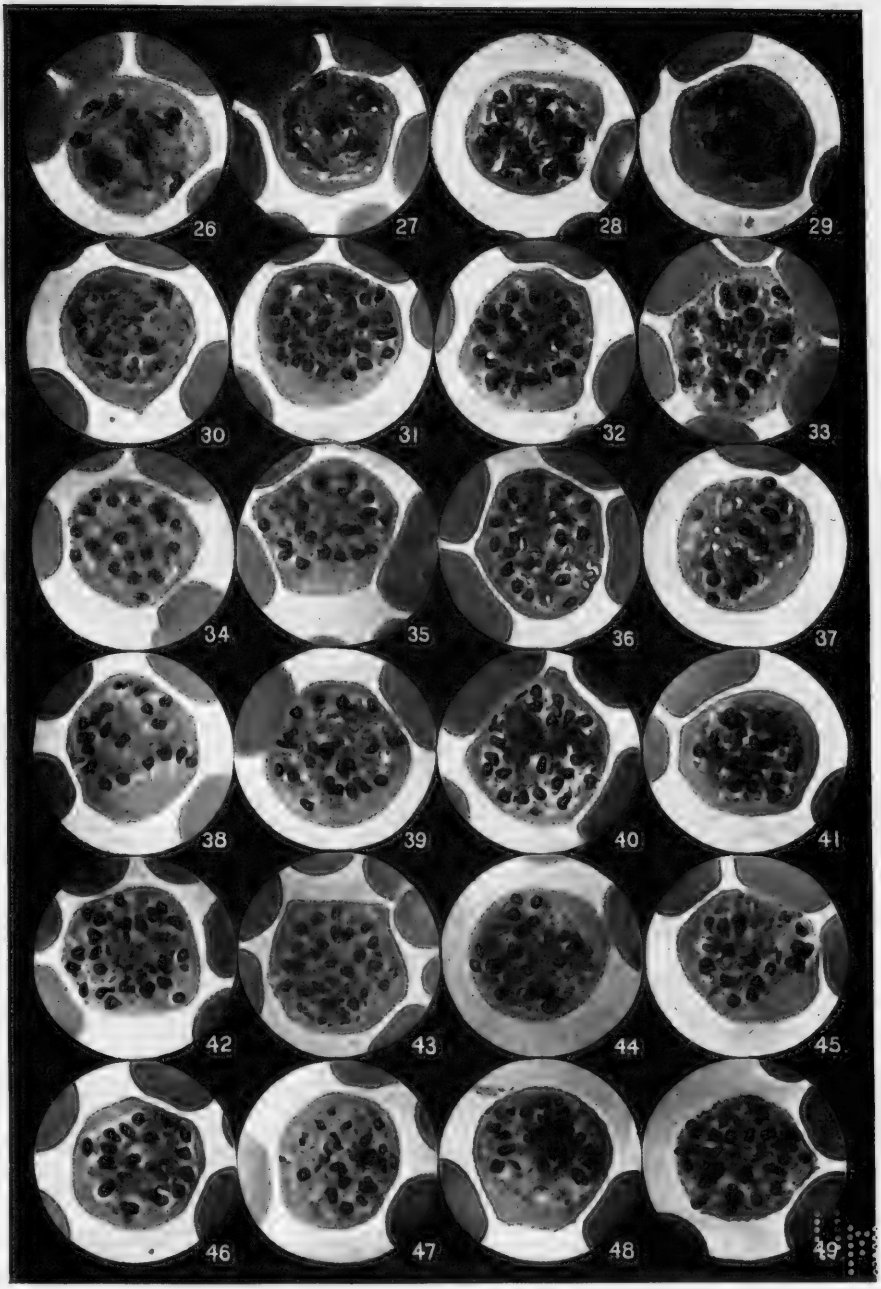
FIGS. 26 to 30. Presegmenting parasites attached to normal red corpuscles which are enlarged by the large size of the attached parasites.

FIGS. 31 to 33. Segmenting parasites attached to red corpuscles which show a slight alteration due to parasitic action. Note the fine stippling of the cells (Shüffner's granulation).

FIGS. 34 to 49. Segmenting parasites attached to fairly healthy red corpuscles. Note the large number of segments of some of the segmenting bodies.



(Lawson: Tertian malarial parasites.)



(Lawson: Tertian malarial parasites.)

BLACKHEAD IN CHICKENS AND ITS EXPERIMENTAL PRODUCTION BY FEEDING EMBRYONATED EGGS OF HETERAKIS PAPILLOSA.

By THEOBALD SMITH, M.D., and H. W. GRAYBILL, D.V.M.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, May 19, 1920.)

The occurrence of blackhead or typhlitis in chickens has been noted by several observers. To the meager literature reported by Smith[1] in 1915 the following may be added.

In 1907 Theobald[2] reported the occurrence of the disease in England. In 1908 Milks[3] observed it in four widely separated localities in Louisiana. It was not seen in birds more than 6 weeks old. The course of the disease was rapid and the mortality 30 to 50 per cent. Higgins[4] found in one chicken ulceration of one cecum which he describes as typical of enterohepatitis or blackhead in turkeys. Tyzzer[5] observed the disease in two chickens. In a chick 4 weeks old there was found a slight invasion of the cecum and extensive involvement of the liver. A slight involvement of one cecum was later found in a hen 2 years old.

During the past 3 years a few cases of disease in chickens associated with *Amœba meleagridis* were brought to the laboratory. In the brief notes given below Nos. 18 and 19 were from one flock, and Nos. 91 and 92 from another. These flocks were over 50 miles apart.

Chicken 18, White Leghorn, brought alive Apr. 18, 1917. Said to have been hatched in Feb. At the autopsy there was found an intussusception of the small intestine of which about 7 cm. were involved. This portion was in a hemor-

[1] Smith, T., *J. Med. Research*, 1915, xxxiii, 243.

[2] Theobald, F. V., *2nd Nat. Poultry Conference, Official Rep.*, Reading, 1907, 181.

[3] Milks, H. J., *Louisiana Agric. Exp. Station, Bull. 108*, 1908, 1.

[4] Higgins, C. H., *Dom. Canada, Dept. Agric., Health of Animals Branch, Bull. 17*, 1915, 1.

[5] Tyzzer, E. E., *J. Med. Research*, 1919, xl, 1.

rhagic condition. Each cecum contained a consistent fecal mould coated with a thin but tough, partly white, partly grayish exudate. The walls of the ceca were thickened, opaque. The liver contained quite small, scattering, indistinctly outlined, yellowish foci. Sections of the ceca showed complete loss of the mucosa which was replaced by a dense layer of fibroblastic tissue. Below this layer in the muscular coat and extending to the serous covering, there were numerous vacuoles of various sizes in the tissue which contained each from one to a dozen or more roundish bodies about 6μ in diameter. Each contained a nucleus with a minute deeply stained caryosome in state of binary division in some of the organisms. In the liver sections the foci seen at autopsy were about one-third the diameter of a lobule. They consisted of necrotic liver cells, parasites like those of the cecal walls, chiefly within giant cells, and many lymphoid elements.

Chicken 19, White Leghorn, received at the same time and from the same flock. The ceca were affected as in No. 18, but the liver was free from foci. Sections of the ceca showed the same kind of changes as in No. 18, but portions of the mucosa were still present. Parasites were rare and seen only in some of the many vacuoles scattered through the superficial fibroid substitute for the destroyed parts of the mucosa. These vacuoles probably lodged parasites now destroyed.

Chicken 91, Rhode Island Red, had been killed before reaching the laboratory. Female, weighing 500 gm. Both ceca contain consistent cores of fecal material which are not adherent to the wall. The underlying mucosa is smooth and free from necrotic areas. The liver is enlarged and covered with opaque whitish foci 6 to 7 mm. in diameter. Some contain minute yellowish specks in the whitish ground. In teased preparations from these foci numerous homogeneous, roundish bodies, about 12μ in diameter, are seen. These are not distinguishable from *Amœba meleagridis* found in diseased turkeys. In sections of the ceca, the thickening of the wall is due to a marked increase of plasma-like cells in the intertubular tissue. Small groups of *Amœba meleagridis* are scattered through the mucosa and one large group is located in the muscular portion of the intestinal wall. Coccidia are scattered through the core. Sections of the liver contained large numbers of *Amœba meleagridis*. This organ was involved as extensively and intensively as in fatal cases of blackhead in turkeys, while the lesions of the ceca were slight.

Chicken 92, Rhode Island Red, was received dead from the same flock. In this bird coccidiosis predominated and the only traces of blackhead found were two microscopic foci in the liver tissue crowded with *Amœba meleagridis*.

From the preceding quotations and studies it is obvious that fatal blackhead is relatively rare in chickens. The data indicate that it may be a quite common disease which passes unnoticed because partial or total recovery is the rule. The injury to the mucous membrane as shown in Cases 18 and 19 may prevent the recovered bird from reaching normal full development. The lesions and the asso-

ciated parasite appear to be identical with those observed in turkeys. The disease shows, however, greater variations from case to case. In some the liver is the chief seat and the ceca almost intact. In others the reverse is true.

The Experimental Production of Blackhead.

Following the successful attempts to produce blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*,[6] similar experiments were made on chickens.

Experiment 1.—The chickens were hatched in an incubator. The eggs before incubation had been washed to remove any adhering dirt, placed in 0.5 per cent bichloride of mercury for 30 seconds, washed again, and dried. During the experiment the chicks were kept in brooders within isolation units, protected against vermin and infectious material likely to reach them if kept in the open. The only source of infection to which they were exposed was the grain and sour milk fed regularly with cooked food.

The worms were obtained from the ceca of two adult hens penned with old turkeys in an outdoor enclosure since the fall of 1919. To liberate the ova, the females were cut up in a Petri dish containing a shallow layer of physiological salt solution. In this they were incubated at room temperature. The feeding was done Mar. 1, when the cultures were 14 days and the chicks 32 days old. Four White Leghorns, four Plymouth Rocks, and four Rhode Island Reds were fed the ova mixed with the food in cages. After the feeding their feet were thoroughly washed and they were placed in a brooder in an isolation unit. From the same hatch nine chickens were held as controls in another unit. The chickens were killed and autopsied at certain intervals after the feeding. In case the ceca were diseased, one was opened and examined as to contents and condition of the mucosa and the other simply incised and then placed in Zenker's fluid for future study.

Chickens killed respectively 1, 2, 3, and 7 days after feeding were found normal, except for weakness of the legs, due presumably to confinement. Sections of one cecum and of liver tissue showed normal conditions. The contents of the ceca consisted of bacteria, a few food remnants, and some *Heterakis* larvæ. The walls were free from infiltrations and contained only the usual number of lymphoid cell groups. In the livers a few small compact groups of cells resembling lymphocytes were present.

In No. 279, killed 10 days after feeding, both ceca were distended and contained a firm reddish core replacing the usual soft contents. The walls of the nonvillous portion were quite uniformly thickened to about 1 mm. Larval worms

[6] Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, xxxi, 647.

(*Heterakis papillosa*) were found in the lumen. The liver was of normal appearance. Transections of one cecum fixed without disturbing the core showed a number of changes. The core, replacing the normal fecal mass, consisted almost wholly of red blood cells, only a few of which had retained the hemoglobin. There were also some necrotic tissue cells in the mass. Throughout the clot were small colonies of bacteria and a few larval forms of *Heterakis*. The mucosa was greatly altered. In several places it was completely destroyed. Elsewhere, the tubules were scarce. They were distorted and oblique to the surface. The mucosa and submucosa contained large groups of lymphocytes and strands of the same were lying in the muscular tissue. Parasites resembling *Amœba meleagridis* were abundant in the mucosa and submucosa. Many were within giant cells. The muscular coats were free. Sections of a small nematode were found in the intertubular tissue, measuring about 0.05 mm. in diameter. In the liver there were fairly numerous dense collections of lymphocytes up to 0.15 mm. diameter. Parasites could not be detected in these foci.

No. 280, killed 11 days after feeding, presented the same lesions. Neither this nor the preceding bird had shown evidences of disease. The core in the cecum was like that of No. 279. The wall presented certain differences, however. The epithelium and the tubules were normal and intact. The submucosa was markedly edematous and the lymphoid tissue somewhat increased. The blackhead parasite had permeated quite generally and distended the intertubular tissue of the mucosa and had penetrated into the submucosa in large numbers. Worms were seen in the mucosa and the lumen. The cell foci as described under No. 279 were present in the liver of this case.

Chicken 281 was killed 14 days after feeding ova. It had not shown signs of illness. The contents of one cecum were normal and contained larval worms. The wall was possibly slightly thickened and the mucosa sprinkled with minute hemorrhages. Sections of the other cecum showed conditions differing both from the preceding and the following case. The contents were normal. A small portion of the wall was normal, the rest thickened. The tubules and surface epithelium were intact. One larval worm was found partly embedded in a tubule. The increased thickness of the wall was due chiefly to a great increase in lymphoid cell groups in the submucosa. In the mucosa there was a slight diffuse infiltration of plasma cells. The muscular coat was not involved. *Amœba meleagridis* occurred in groups of two to six or more individuals in tissue spaces. The parasites were relatively scarce as compared with No. 279. The liver contained minute collections of lymphoid cells, from one to two in a field of the 16 mm. objective.

Chicken 282 was killed 15 days after feeding. It had not appeared quite normal. Both ceca were distended and firm to the touch, owing to the presence of hemorrhagic cores replacing normal feces. The walls were 2 to 4 mm. thick. Larval worms were present. The core was made up of a mass of red corpuscles embedded in a homogeneous feebly stained matrix. In one spot it was incorporated with the wall and here the mucosa was destroyed. The rest of the mucosa was covered with epithelium but the tubules were partly destroyed, partly dis-

torted. The intertubular tissue was infiltrated with lymphoid cells and amebæ. The submucosa was markedly edematous. At irregular intervals it contained large dense collections of cells enclosing amebæ. In a few places the muscular coat was infiltrated with lymphocytes.

Chicken 285, killed 18 days after feeding, had not shown symptoms of illness. It was normal as to ceca. There were a few whitish foci 1 to 2 mm. in diameter in the liver. Transverse sections of fixed and hardened tissue from four different levels of one cecum showed the same changes. There were a cellular infiltration and increase of lymphoid tissue in the mucosa and a few tubules were markedly distended. Just below the muscularis there was a dense zone or layer of cells, probably lymphocytes, and roundish masses of the same, suggesting newly formed follicles. There was no core or mass of exudate in the lumen and there were no parasites in the tissues. In the liver sections, besides the minute collections of lymphoid cells, there are a few larger groups of the same type of cells enclosing necrotic liver tissue. *Heterakis* larvæ were in the ceca.

Chicken 286, killed 23 days after feeding, had not shown symptoms. The contents of the ceca were normal and larval worms were present. There was evidence of increased numbers of cells, resembling plasma cells in the mucosa. Lymphoid follicles were more numerous than normally in the submucosa, but the diffuse infiltration of lymphocytes was lacking.

Chicken 287 was killed after 28 days. It had not shown symptoms. One cecum contained some *Heterakis* larvæ, but contents were normal. Sections of the other cecum showed slight irregularity and occasional loss of tubules, their places being filled with cells of lymphocyte type. In the intertubular tissue there was an increase of plasma-like cells. The submucosa contained more than the normal number of lymph follicles. There was one group of eighteen follicles. In the liver certain whitish foci, 1 mm. in diameter, seen at the autopsy, were found to be made up entirely of cells of lymphocyte type. A central mass about 0.5 mm. diameter was enclosed in a ring of follicles. Many small foci of similar cells, 0.1 to 0.15 mm. diameter, frequently filling out vessels were also present.

Chicken 288 was killed after 29 days. *Heterakis* larvæ in ceca. Although the animal had shown no distinct signs of disease and the organs appeared normal with the exception of a few whitish areas on the liver 1 to 2 mm. in diameter, the walls of the ceca were not normal. The mucosa had the usual number of tubules with the exception of about one-tenth of the circumference, in which the tubules were replaced with lymphoid cells. There was a general increase of cells of plasma type between the tubules and the number of follicles in the submucosa was increased. In a section of the liver there were two foci, one consisting of a plug of cells in a vessel, the other a group of cells simulating four lymph follicles surrounding a small vessel also plugged with lymphoid cells.

Putting the data of this experiment together, we observe that as a result of hemorrhages the contents of the ceca appeared as firm cores

on the 10th to the 15th day inclusive, but not before the 10th, nor after the 15th day. Protozoan parasites were in the walls of the ceca within the same period. A larger number of cases examined at shorter intervals will define these limits more accurately.

Of the controls, which had remained well with the exception of some lameness due to confinement, two were killed 17 days after the other lot had been fed, and one 30 days after. None showed signs of lesions and worms were not found.

Experiment 2.—In this experiment, which is similar to Experiment 1, certain controls were introduced. The chickens were obtained from a poultry farm just after they had been hatched and placed in a brooder in an isolation unit. They were fed as were those in Experiment 1. The worms used were obtained from the same source as those used in Experiment 1. The cultures were prepared in the same way as heretofore. Three groups were included, each consisting of four White Leghorn chickens. Group I was fed with cultures made by cutting up adult female *Heterakis* to permit eggs to escape. Group II received cultures prepared by cutting up only males. Group III was fed with sediment from the washed contents of the ceca after the worms had been removed and the contents run through a wire screen of No. 300 mesh which did not permit the few free ova to pass through. At the time of feeding, the cultures were 20 days and the chickens 18 days old. All of the chickens in Group I were diseased, as the following notes show.

On the 9th day after feeding No. 290 was not quite normal. Its head was drawn back and its wings drooped slightly. It was chloroformed next day. The body was in good condition and only the ceca were affected. They were distended to about 1 cm. in diameter, firm to the touch, and hemorrhagic at the distal end. One, cut open, contained a spongy pink and reddish core slightly adherent to the hemorrhagic mucosa. The liver showed some whitish specks. Transverse sections from the other cecum showed the presence of a core made up of a meshwork of a homogeneous substance containing some red corpuscles. It was attached to the greater part of the wall and the mucosa was here destroyed. The remaining mucosa was low, with continuous epithelium and distorted tubules. The submucosa was broadened by infiltration with large numbers of *Amœba meleagridis* and lymphoid cells. The muscular layers were also infiltrated with cells, even into the mesentery. Larvæ were present in the mucosa and lumen. In the liver sections only minute groups of lymphocytes were present.

Chicken 295 was not quite normal on the 11th day, when it was chloroformed. The gross appearance of the ceca and the condition of the wall and core of one cecum as shown in sections were so like those of No. 290 that a detailed statement is omitted. In the liver a necrotic focus with a few *Amœba meleagridis* in it deserves mention.

Chicken 296 was killed on the 12th day. It had not shown any distinct signs of illness. Both ceca were distended and firm. One was opened and a long, cylindrical, non-adherent core, gray and brown in color, removed. The wall was about 2 mm. thick. In stained sections, the core was found composed chiefly of exudate cells (lymphocytes). It was attached at one spot to the wall and here the mucosa had been destroyed. Elsewhere the low mucosa was covered with epithelium. Normal tubules were scarce. The rest were much dilated and filled with cell debris. The mucosa, submucosa, and muscular layers were indistinguishable, owing to a general invasion of *Amœba meleagridis* and a general infiltration of cellular elements, lymphocytes, and plasma cells. There were also in the muscular coat masses of cells simulating lymph follicles. In the liver there were the usual minute collections of lymphocytes and some necrotic foci, but parasites were not seen in them.

The 4th and last chicken of Group I, No. 298, was killed 13 days after feeding. It had been clinically normal. Both the macroscopic and microscopic pictures were so like those of No. 296 that no detailed description is given. The invasion of *Amœba meleagridis* was slight below the submucosa, but the infiltration of the musculature with large masses of lymphocytes, even into the mesentery, was striking.

In three of the foregoing cases larval worms were found in the contents of one cecum when examined fresh. In the fourth they were demonstrated in transections of the core of the other cecum.

The four chickens of Group II remained normal until they were killed on the 10th, 11th, 13th, and 13th day, respectively. No lesions were found and worms could not be detected. The same was true for the chickens of Group III, which were killed on the 10th, 11th, 12th, and 13th day, respectively.

DISCUSSION.

The foregoing experiments demonstrate that a disease of the ceca very closely resembling that of the enterohepatitis or blackhead of turkeys can be produced in chickens still in the brooder by feeding an overdose of embryonated eggs of *Heterakis papillosa*. The lesions appeared on or about the 9th day after feeding. Chickens killed after 15 days showed, in spite of an intact mucosa, signs of a past inflammation in the presence of large numbers of cells of the lymphocyte type in mucosa and submucosa and in the increased number of roundish accumulations of similar cells resembling lymph follicles.

The active disease manifests itself in a pouring out of blood into the lumina of the ceca, which coagulates into a firm spongy core. Later the red corpuscles disappear and lymphocytes emigrate from the

injured mucosa to form the outer zone of the core. This is attached in part to the wall and here the mucosa is necrotic. The walls of the ceca are thickened up to 4 mm. in diameter. The thickening is due to edema, infiltration of lymphocytes and of *Amœba meleagridis* in large numbers. The mucosa is more or less injured in addition to the necrosis where the core is adherent. The tubules are distorted, dilated, and in part missing. Some are filled with cell debris. In all cases there is a diffuse infiltration of plasma-like cells between the tubules. Occasional hemorrhages into the substance of the mucosa are present.

The liver, besides containing in all cases microscopic focal collections of lymphocytes, shows rather infrequently barely visible yellowish specks which consist either of necrotic foci in which giant cells and cells of endothelial type are replacing the liver cells or else of collections of lymphocytes in the form of roundish follicles or within vessels. *Amœba meleagridis* was seen in one case in a necrotic focus. On the whole, the changes in the liver so formidable in turkeys are insignificant.

The protozoan parasites presented the same morphological characters shown in the turkey's tissues. They occupied the tissue spaces in mucosa and submucosa and more rarely in the muscular coat. As a rule, the bodies of the parasite appeared as if in a state of disintegration except in No. 281, in which they had a homogeneous cytoplasm. In some cases most of them were within phagocytic cells. In one case (No. 290) the protozoa set free from teased portions of the mucosa showed, without the use of a warm stage, finger-like pseudopodia in continual change.

The genesis of the clot in the ceca is not clear. The cases studied were not timed so as to encounter it in its formation. This gap must be filled before any basis for a discussion of the nature and significance of the cecal lesions can be found.

In general the larval stages of *Heterakis* were present but in small numbers. In four cases individuals were detected in the mucosa itself.

Flagellates so common in the tubules of the turkey were entirely absent in the two series of chickens.

The injurious effects of feeding embryonated eggs of *Heterakis papillosa* to young chickens are no less definite than those observed after feeding them to turkeys. There are, however, certain distinctions to be drawn between the disease in turkeys and in chickens. The turkeys were found much more susceptible since the feeding produced uniformly a severe disease probably fatal in all birds if those that were chloroformed had been allowed to live longer. The effect on the condition of the young chickens was slight and they would probably have all survived.

The lesions due to the feeding differ materially as regards the liver. In turkeys this organ is almost uniformly invaded by *Amœba meleagridis* and the resulting foci of multiplication lead to a destruction of a variable amount of liver tissue, often over 50 per cent. In chickens the invasion is so slight that when it does take place the resulting lesions are scarcely more than microscopic in size. The liver is not wholly immune, however, as spontaneous cases now and then prove.

As to the ceca, the lesions induced in chickens appear formidable enough, but they probably undergo speedy resolution and the destroyed mucosa is covered with epithelium in due time. The after effects of the partial destruction of the mucosa may be more serious and tend to interfere with the normal growth of the chicken. This destruction may be permanent and lead to a replacement of the mucosa by scar tissue, as shown in the two spontaneous cases described above. In general it may be assumed that injury to the ceca due to *Heterakis* and *Amœba meleagridis* is not uncommon and its causal relation to other pathological conditions of poultry may be far reaching. Not until this worm has been largely suppressed can the extent of the injury due to it be inferred in retrospect. The degree of injury inflicted appears to be largely a question of dosage. The more ova ingested the more widespread and intensive the lesions, whereas the ingestion of a few does not appear to be dangerous even to turkeys.

Indications of a tendency of the nematodes to encyst in the walls of the ceca, as observed by Letulle and Marotel[7] in a pheasant, were wholly absent in the cases examined.

The presence of *Amœba meleagridis* in chickens still in the brooder after they have been fed ova either free or still within fragments of

[7] Letulle, M., and Marotel, *Arch. parasitol.*, 1908, xii, 361.

worms incubated in physiological salt solution for 15 to 20 days at room temperature points to the presence of *Amœba meleagridis* in the cultures. This cannot be confirmed or disproved until some method other than feeding *Heterakis* eggs is found which will induce conditions favorable to the invasion of *Amœba meleagridis* into the walls of the ceca. So far it has been impossible to start the disease by feeding incubated feces or cultures from which the worms and ova had been removed. It should be stated that protozoa resembling amebæ and flagellates have been found in the cultures of ova fed and a study of these is now under way. The relation of *Heterakis papillosa* to typhlitis in turkeys and chickens and the slightly varying morphology of the invading protozoa present for consideration the possibility that the latter may not necessarily belong to one species.

CONCLUSIONS.

Feeding embryonated eggs of *Heterakis papillosa* to brooder chickens led to a disease of both ceca, characterized by the presence of a core consisting of fecal matter, coagulated blood, and emigrated cells from the mucosa. The walls of the ceca were thickened as a result of cell invasion and multiplication, invasion and multiplication of *Amœba meleagridis* or allied parasites, and more rarely hemorrhage and edema. The respective parts played by *Heterakis papillosa* and the protozoa in starting the lesions and the source of the protozoa remain to be defined. The invasion of the liver by the protozoa was insignificant.

EARLY CHANGES FOLLOWING THE INJECTION OF TUBERCLE BACILLI INTO THE METAPHYSIS OF THE LONG BONES OF ANIMALS.

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PLATE 4.

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It has long been recognized that tuberculous infection of long bones originates and is in great part confined to the metaphysis and epiphysis. There is a marked contrast in this localization to the diffuse process which follows infection with pyogenic organisms, and it is not surprising that many conjectures have been made to explain the observed facts. Notable among these is the suggestion of Lexer,[1] whose anatomical studies led him to believe that embolic deposit of bacteria in the region of the metaphysis is favored by the course of the arteries. On the other hand, Ely[2] claims that peculiarities in the structure of the bone marrow more clearly explain the facts.

The following experiments were done to examine the latter factor. No attempt was made to infect the bone in a manner analogous to spontaneous infection in man, as our interest is concerned with the cellular reaction which follows the infection, rather than with the path of the infection. As an aid in the determination of the various cell types the animals were vitally stained.

In normal vitally stained animals the leucocytes and the megacaryocytes show no deposits of the dye. The fibroblasts are as a rule also free from dye, though in intensely stained animals a few fine granules are seen at either end of the nucleus. The cell that shows the vital stain in greatest amount is the reticulo-endothelial cell which lines the venous spaces of the marrow. The protoplasm of

[1] Lexer, E., *Arch. klin. Chir.*, 1904, lxxiii, 481.

[2] Ely, L. W., Joint tuberculosis, New York, 1911.

these stellate or triangularly shaped cells is crowded with bright blue granules of varying size, so that the nucleus may be entirely hidden. It is these cells which have been described as phagocytosing blood pigments, and as normally possessing a brown pigment.[3] The reaction of these cells to the vital stain has caused them to be included in the large group termed histiocytes,[4] or resting wandering cells.[5] They correspond roughly to the macrophages of Metchnikoff.

The histogenesis of the tubercle has been studied with the aid of vital staining by several investigators. Oppenheimer,[6] Goldmann,[7] and Evans, Bowman, and Winternitz[8] have used this method in following the development of the tubercle in the liver, and Joest and Emshoff[9] in the lymph glands. Kiyono mentions the bone marrow,[4] but gives no details of his observations. All these investigators agree that the early groups of epithelioid cells forming the miliary tubercle are made up of, or derived from the vitally stained histiocytes found in these organs.

Technique.

Rabbits were given 20 cc. of a 1 per cent solution of trypan blue intravenously, and 2 days later, a trephine opening[10] was made in the metaphysis of the tibia and one to two drops of a normal salt solution emulsion of bovine tubercle bacilli injected into the marrow. Similar experiments were made with guinea pigs and tubercle bacilli of the human type. After varying intervals, from 3 to 9 days, the animals were killed and portions of the bone removed and fixed in 10 per cent formaldehyde. After fixation the marrow was removed from the bone, embedded in paraffin, and sections were appropriately stained.

[3] Brass, H., and Weidenreich, F., *Arch. mikr. Anat., 1te Abt.*, 1913, lxxxii, 61.

[4] Kiyono, K., Die vitale Karminspeicherung, Jena, 1914.

[5] Tschaschin, S., *Folia hæmatol.*, 1913–14, xvii, 317.

[6] Oppenheimer, R., *Virchows Arch. path. Anat.*, 1908, cxciv, suppl., 254.

[7] Goldmann, E. E., Neue Untersuchungen über die Äussere und Innere Sekretion des gesunden und kranken Organismus in der "vitalen Färbung," Tübingen, 1912, 67.

[8] Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.

[9] Joest, E., and Emshoff, E., *Virchows Arch. path. Anat.*, 1912, ccx, 188.

[10] The operation was performed under ether anesthesia.

In all the animals so treated it was possible to demonstrate either the bacilli, definite tubercles and giant cells, or diffuse caseation, depending on the length of time that the animal lived after infection. Guinea pigs proved more satisfactory for the early stages, as the bovine bacilli used in the experiments on rabbits were difficult to stain.

EXPERIMENTAL.

In our experiments the earliest changes were observed 3 days following the operation. No lesion could be seen in the gross, and but little change was observed in sections with a low magnification. Around the area of injection, which showed little or no evidence of trauma, the lymphoid cells were decreased in number, while the vitally stained reticulo-endothelial cells were more numerous than in the normal marrow at a distance from the trephine opening.

With a higher magnification it was seen that a large number of the leucocytes showed evidences of nuclear degeneration, while the reticulo-endothelial cells contained two or more nuclei, and instead of being stellate in shape as normally, were now rounded and lay free in the sinuses of the marrow (Fig. 1). No mitotic figures were observed in these cells but the increase in their number and the presence of more than one nucleus to a single protoplasmic body warrant the assumption of an active proliferation on their part.

Another appearance noted in these cells was the presence of clear areas free from dye granules which showed no coloration with the counterstain. These were interpreted as vacuoles, and in sections stained for tubercle bacilli it was possible to demonstrate in them one or more acid-fast bacilli. With an increase in the number of bacilli in the cell there was a corresponding decrease in the number of dye granules. Fig. 2 shows a cell filled with bacilli and having many large vacuoles and a few faded dye granules.

In somewhat later stages of the infection groups of vitally stained reticulo-endothelial cells were seen consisting of from ten to twenty cells (Fig. 3). These cells contained acid-fast bacilli and showed varying degrees of the changes described above. It was impossible to determine whether these cells had proliferated *in situ* or wandered to the point of infection. The impression that the latter is the case

is obtained from the appearance of the cells, though both methods of increase doubtless occur.

A few giant cells were seen in the sections. These had the typical morphology of the Langhans type (Fig. 4). In their clear protoplasm could be seen dye granules and vacuoles, which in suitably stained specimens showed acid-fast bacilli. In many instances the contours of the individual reticulo-endothelial cells forming them could still be made out.

No attempt was made to follow the tuberculous process in its further development. Several animals were allowed to live a month or until death. In all these animals extensive tubercular lesions were found in the diseased bones, consisting of broad areas of caseation surrounded by granulation tissue. In this granulation tissue were many reticulo-endothelial cells still showing dye granules.

The demonstration by Lexer of numerous anastomoses in the metaphyses of long bones, though it may explain the more frequent embolic deposit of bacteria in that region, does not explain the difference observed in the localization of a tuberculous process, as contrasted with the diffuse lesion seen in pyogenic infections.

The present experiments emphasize the view that the observed difference may be due to peculiarities in the structure of the infected tissue, the bone marrow, for this tissue in the metaphyses of long bones as contrasted with the fatty marrow of the diaphyses, is rich in the cells which are particularly concerned in the reaction to infection with tubercle bacilli.

I wish to express my thanks to Dr. L. W. Ely and Dr. F. E. Blaisdell for their aid in the operations on the animals.

EXPLANATION OF PLATE 4.

All the figures were drawn with the aid of a camera lucida. Bausch and Lomb ocular 1, objective $\frac{1}{12}$.

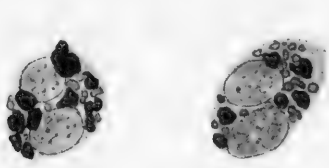
FIG. 1. Two reticulo-endothelial cells from the marrow of a vitally stained rabbit infected 4 days previously with bovine tubercle bacilli. The dye granules are of normal appearance, but each cell body contains two large nuclei. Carmine counterstain.

FIG. 2. Reticulo-endothelial cell from the marrow of a guinea pig 6 days after infection. Many vacuoles are seen and a large number of phagocytosed tubercle bacilli. The dye granules are few in number and pale. Stained with carbol-

fuchsin, decolorized with acid alcohol, and counterstained with methylene blue.

FIG. 3. A group of vitally stained cells in the marrow of a guinea pig 9 days after infection. The cells show varying degrees of vacuolation and six of them contain tubercle bacilli. The same stain as in Fig. 2.

FIG. 4. The same specimen as Fig. 3. A typical Langhans giant cell with dye granules and vacuoles in the central protoplasm. Carmine counterstain.



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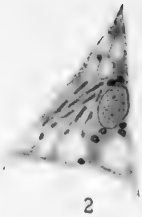

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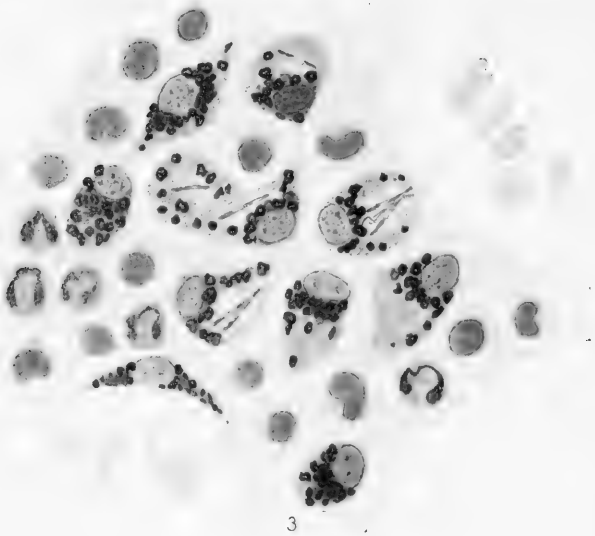

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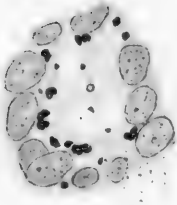

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(Oliver: Tubercle bacilli.)

IMPORTANCE OF BLOOD GROUPS IN COMPLEMENT FIXATION REACTIONS.

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(Received for publication, April 7, 1920.)

The complement fixation test for syphilis has become one of the most valuable and widely applied of diagnostic reactions, in spite of the empirical nature of several features of its technique. Increasing experience has eliminated several sources of technical error, and progress is being made toward standardization and greater reliability. One of the well known sources of error in the original Wassermann test is the possible presence of an excess of amboceptor in the form of natural anti-sheep hemolysin in human serum. That this excess of amboceptor is of importance seems to have been proved by many workers. The recent results obtained by Kolmer and Rule (1) are especially conclusive.

In this paper attention is called to a similar possible source of error with the use of an anti-human hemolytic system. It is shown that an excess of amboceptor may result from a natural hemolysin in the guinea pig complement and from the isolysins in human serum, when the blood cell suspensions are made up at random. A simple way of avoiding this possible inaccuracy is pointed out, which consists in the use of a certain human blood group in the preparation of the human cell suspensions. Blood groups among sheep seem to be less clear and less important than among human beings.

The remark has occasionally been made by different serologists that, theoretically, the human blood groups might be of importance in complement fixation work with the human system. Search of the literature, however, has failed to reveal any instance of a practical application of this theory. The only statement found which directly bears on this subject is in War Manual No. 6, Laboratory methods,

U. S. Army (2), which gives the theoretical possibilities but makes no mention of the actual importance of blood groups, and no practical application of this theory had been made in this laboratory until the present study was completed. In a recent article Kolmer, Trist, and Flick (3) report their results with human isolysins in serological work, but they make no effort to apply the results practically, and, from the standpoint of this paper, their experiments are not conclusive, for while they use several different cell suspensions against different human sera, no statement is made as to the blood groups to which each cell suspension belonged. That this is of major importance will be shown later.

As the work reported here was undertaken for practical information, the technique followed closely the method of Craig (4), which is in routine use at the Army Medical School. 0.1 cc. of the inactivated human serum was always used; the guinea pig sera and the pooled complement were always diluted to 40 per cent and the dose was expressed either in units or actual quantities. These quantities were held constant in all the work with an anti-human hemolytic system. The work with the anti-sheep hemolytic system was performed with the quantities, dilutions, and dosage used in what is commonly known as the quarter unit Wassermann test.

The human blood groups were designated according to the classification of Moss (5) as follows:

Group I. Serum agglutinates the cells of no other group.
Cells are agglutinated by sera of Groups II, III, and IV.
Group II. Serum agglutinates the cells of Groups I and III.
Cells are agglutinated by sera of Groups III and IV.
Group III. Serum agglutinates the cells of Groups I and II.
Cells are agglutinated by sera of Groups II and IV.
Group IV. Serum agglutinates the cells of Groups I, II, and III.
Cells are agglutinated by no other sera.

Moss (5), Grafe and Graham (6), Zinsser (7), and many others have made the observation that the isohemolysins and isohemagglutinins are practically parallel in the human blood groups. These observations have resulted in the formulation of a phrase which has become axiomatic in human blood group work preliminary to blood transfusion—hemolysis never occurs when agglutination is absent. It is believed that this phrase can be amplified to include the statement that it is always possible to obtain hemolysis when agglutination is present. In as far as this applies to the human blood groups only, the work reported here amply cor-

roborates the truth of both statements. Kolmer and his coworkers (3) state that the presence of hemagglutination and hemolysis bears no direct relation to each other. Their experiments are open to the same criticism as before, in that they were working with various sera and cells, the blood groups of which were unknown. It is possible that their failure to obtain hemolysis when hemagglutination was present was due to an insufficient amount of complement. The two instances in which they obtained hemolysis when no macroscopic agglutination was present is harder to explain and has never been encountered in our work with the human blood groups.

A failure to obtain hemolysis in group cells or by group sera, the groups of which had previously been determined by the agglutination method, was never encountered in this work, nor was hemolysis ever obtained when agglutination was absent. It is true, however, that, quantitatively, the hemolysin content of a human serum does not necessarily correspond to the agglutinin content. A serum giving strong agglutination for a certain group of cells may give slight or no noticeable hemolysis, in the quantity used, against the same cells. The hemolysin is present, however, and can always be demonstrated, when sufficient complement is present, by increasing the amount of serum.

It seems correct, therefore, to substitute the word "hemolyzes" for "agglutinates" in Moss' classification of the human blood groups; *i.e.*,

Group I. Serum hemolyzes the cells of no other group.
Cells are hemolyzed by sera of Groups II, III, and IV.
Group II. Serum hemolyzes the cells of Groups I and III.
Cells are hemolyzed by sera of Groups III and IV.
Group III. Serum hemolyzes the cells of Groups I and II.
Cells are hemolyzed by sera of Groups II and IV.
Group IV. Serum hemolyzes the cells of Groups I, II, and III.
Cells are hemolyzed by the sera of no other group.

Upon the above premises the work upon the influence of the human blood groups in complement fixation reactions was undertaken.

Presence of a Natural Specific Hemolysin in Guinea Pig Serum for Group I and Group II Human Cells.

Trouble has been experienced at various times in our complement fixation work by encountering complement that was hemolytic for the particular cell suspension being used. This was never a constant factor and would often be absent or at least not noticeable in the control tube containing complement and cells in the complement titration. The presence of this hemolysin was particularly noted in France while using an anti-sheep hemolytic system, and at one time became a serious problem, warranting the issuance of a special circular by the Director of the Division of Laboratories and Infectious Diseases warning all laboratories to be on the watch for it and suggesting that the serum of each guinea pig should be tested against the cell suspension used that day, before the sera were pooled for use as complement. Certain investigations were undertaken at this time under the direction of Major R. G. Hussey and with the benefit of occasional advice from Colonel Zinsser. The pressure of routine work was so great, however, that nothing definite was accomplished.

In some of the experimental work in France it was shown that the serum of these guinea pigs often contained hemolysins for both human and bovine cells, although these hemolysins were not constant and did not parallel the natural anti-sheep hemolysin present. With this fact in mind, since changing to the anti-human hemolytic system, I have tested out a portion of each guinea pig serum, before it was pooled as complement, against the human cell suspension being used that day. It was often observed and demonstrated that on certain days practically all the guinea pig serum would contain a sufficient quantity of anti-human hemolysin to render it unfit for use as complement. On these occasions many guinea pigs would have to be bled before it was possible to find three or four sera suitable for pooling and use as complement.

While engaged in some work with the four groups of human cells it was observed, in certain control tubes containing only complement and cells, that the tubes containing complement and Group I cells, and complement and Group II cells, showed a marked agglutination of the cells. The control tubes with Group III and Group IV cells

TABLE I.

Demonstration of a Natural Specific Hemolysin in Guinea Pig Serum against the Cells of the Human Groups I and II.

Guinea pig No.	Reaction with 0.1 cc. of a 5 per cent suspension of Group I cells. Amount of 40 per cent dilution of guinea pig serum.				Reaction with 0.1 cc. of a 5 per cent suspension of Group II cells. Amount of 40 per cent dilution of guinea pig serum.			
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.
1					−	+−	+−	+
2					−	−	+−	+
3					−	+−	+−	+
4					−	−	+−	+
5					−	+−	+	++
6					+−	+	++	++
7					−	+−	+	++
8					+	++	++	++
9					+−	+	++	++
10					−	−	+−	+−
11					+−	+−	+	+
12					−	−	−	+−
13					−	−	+−	+
14					+	++	++	++
15	−	+	+	++	−	+	+	+
16	+	++	++	++	+−	+	++	++
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25	+	+	++	++	+−	+	++	++
26	−	+−	+−	+	−	+−	+−	+
27	+	++	++	++	+	++	++	++
28	+−	+	+	++	+−	+	++	++
29	+−	+	++	++	+−	+	++	++
30	−	+−	+	+	−	+−	+−	+
31	+	++	++	++	+	++	++	++
32	++	++	++	++	+−	+	++	++
33	−	−	+−	+−	−	−	−	+−
34	+	++	++	++	+	++	++	++
35	+−	+	++	++	−	+−	+	++
36	+	++	++	++	+	++	++	++

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE I—*Concluded.*

Guinea pig No.	Reaction with 0.1 cc. of a 5 per cent suspension of Group I cells.				Reaction with 0.1 cc. of a 5 per cent suspension of Group II cells.			
	Amount of 40 per cent dilution of guinea pig serum.				Amount of 40 per cent dilution of guinea pig serum.			
	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.
37	+−	+	++	++	+−	+	+	++
38	+−	+	++	++	−	+−	+	++
39	+−	+	++	++	−	+−	+	++
40	+	++	++	++	+−	+	++	++
41	+	++	++	++	+	++	++	++
42	+	++	++	++	+−	+	++	++
43	+	++	++	++	+−	+	+	+
44	+	++	++	++	+	++	++	++
45	+−	+−	+−	+	+−	+−	+−	+
46	+	++	++	++	+	++	++	++
47	+	++	++	++	+−	+	++	++
48	−	+−	+−	+	−	−	+−	+
49	+	++	++	++	−	+−	+	++
50	+−	+	++	++	−	−	−	+−
51	+−	+	++	++	−	−	+−	+
52	+	++	++	++	−	+−	++	++

showed no agglutination. On the assumption that when hemagglutinins were present, hemolysins could usually be demonstrated, a large number of guinea pigs was tested against different human cell suspensions of Groups I, II, III, and IV.

The guinea pig sera were diluted to 40 per cent, which is the strength of the complement as used in the Army Medical School, and increasing quantities, 0.1, 0.2, 0.3, and 0.4 cc., were set up against the cell suspensions of the four human groups. Larger quantities were not used, for the complement unit in the anti-human system generally falls in the neighborhood of 0.04 or 0.05 cc. Quantities as large as 0.2 cc. are seldom if ever used, and for practical purposes it was not considered necessary to use quantities larger than 0.4 cc. The results on over 50 guinea pigs were striking. No hemolysis whatever was obtained with any of the guinea pig sera with Group III and Group IV human cells. The results with Group I and Group II human cells are shown in Table I.

TABLE II.

Natural Hemolysin in Guinea Pig Serum before Absorption with Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	+	+	−	−
	0.2	++	++	−	−
	0.3	++	++	−	−
	0.4	++	++	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE III.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group I Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	−	−	−	−
	0.2	−	−	−	−
	0.3	−	−	−	−
	0.4	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE IV.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group II Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	−	−	−	−
	0.2	−	−	−	−
	0.3	−	−	−	−
	0.4	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE V.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group III Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	+−	+	−	−
	0.2	++	++	−	−
	0.3	++	++	−	−
	0.4	++	++	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

TABLE VI.

Natural Hemolysin in Guinea Pig Serum after Absorption with Group IV Human Cells.

Guinea pig No.	Amount of 40 per cent dilution of guinea pig serum.	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
	cc.				
53	0.1	+	+	−	−
	0.2	++	++	−	−
	0.3	++	++	−	−
	0.4	++	++	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

In view of the fact that these guinea pig sera in the same quantities were absolutely negative for Group III and Group IV cells, the results indicate that there is, apparently, a specific hemolysin in guinea pig serum, in quantities that would be a factor if used as complement selective against two groups of human red blood cells. No hemolysin is present for the other two groups of cells in quantities far in excess of any that might be used in complement fixation reactions with an anti-human hemolytic system.

A fact of some importance is that these examinations were made at different times over a period of several months, that the guinea pigs were obtained from various lots purchased in Pennsylvania, Virginia,

Maryland, and the District of Columbia, and that in all the work the different human group cells were not obtained from any one set of individuals but from a large number of people, the only requirement being that they fell within the proper blood group.

The specificity of this hemolysin was next determined by the absorption, in the cold, of many of the guinea pig sera with the different group cells. A specimen of guinea pig serum, 40 per cent, was tested against the four group cell suspensions. A portion of this serum was then treated with Group I cells, at freezing temperature, for $\frac{1}{2}$ hour, rapidly centrifuged, the supernatant serum pipetted off and retested against all four group cell suspensions. Three other portions of the untreated serum were then absorbed with the cells of Groups II, III, and IV respectively, in the same manner, and all separately retested with the cell suspensions of all four groups. A detailed tabulation of the experiments will not be given here, but the results were invariably constant and sample protocols are shown in Tables II to VI.

These experiments show that the natural anti-human hemolysin present in guinea pig serum can be absorbed by the cells of either Group I or Group II, but is not absorbed by the cells of either Group III or Group IV, thus proving the specificity of the hemolysin. As stated before, these experiments were done many times with unvarying results.

Influence of the Isohemolysins in Human Sera upon Human Cell Suspensions.

The influence of the isolysins in sera received for Wassermann test and their relation to the blood group of the cell suspension used were next investigated.

Twenty-three sera, upon which tests had been run, were selected at random. Group I cells were first selected as being, theoretically, the cells most apt to be acted upon by the largest number of sera. 0.1 cc. of the inactivated serum, 0.1 cc. of the Group I cell suspension, and one unit of complement, as determined for the tests that day, were made up to a total volume of 1 cc. with saline solution and incubated in the water bath at 37°C. for 1 hour. Three of the sera gave complete hemolysis of the cells, nine gave marked hemolysis, six gave

slight hemolysis, while five gave no appreciable hemolysis at the end of 1 hour. The three sera that gave complete hemolysis with Group I cells were then set up with a suspension of Group IV human cells, with the same quantities and technique. No hemolysis took place

TABLE VII.

Influence of the Isohemolysins in Twenty Human Sera on the Four Groups of Human Cells.

Serum No. (0.1 cc.).	Amount of complement (40 per cent).	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
913	1 unit.	++	+	+−	−
914	1 "	+	−	−	−
915	1 "	+	+−	−	−
916	1 "	+	+−	−	−
917	1 "	++	+−	−	−
918	1 "	+	−	+−	−
919	1 "	+	−	−	−
920	1 "	++	−	+−	−
921	1 "	+	−	−	−
927	1 "	+−	+−	+−	−
928	1 "	++	++	+	−
929	1 "	−	−	−	−
930	1 "	+	+	−	−
931	1 "	+	+−	+	−
932	1 "	++	−	++	−
933	1 "	+−	−	−	−
934	1 "	++	−	+	−
935	1 "	+	−	−	−
936	1 "	+	+−	−	−
937	1 "	+	+−	−	−
Controls.					
0	2 units.	A	A	−	−
0	0	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis; A, agglutination.

with the Group IV cell suspension. The results with Group I cells were so suggestive that steps were immediately taken to continue the work with cells from all four of the human blood groups.

Twenty sera were next tested with cell suspensions of all four groups. The results are shown in Table VII. Hemolysis, marked or partial,

took place with practically all the human sera and Group I cells. Variations were shown in their action on Group II and Group III cells, according to the blood group to which each serum belonged. The Group IV cell suspension was not hemolyzed by any of the sera.

TABLE VIII.

Influence of the Isohemolysins in Twenty-Six Human Sera When Complement from Which the Anti-Human Hemolysin Had Been Absorbed Was Used.

Serum No. (0.1 cc.).	Amount of complement (absorbed with Group II cells).	Reaction with 0.1 cc. of a 5 per cent suspension of cells of.			
		Group I.	Group II.	Group III.	Group IV.
1	1 unit.	+−	−	−	−
2	1 "	−	−	−	−
3	1 "	+	+−	+−	−
4	1 "	++	+	++	−
5	1 "	+−	−	+−	−
6	1 "	++	+−	++	−
7	1 "	+	−	+	−
8	1 "	+	−	+−	−
9	1 "	+−	+−	−	−
10	1 "	+	−	+−	−
11	1 "	+	+−	+−	−
12	1 "	+−	−	+	−
13	1 "	++	−	++	−
14	1 "	+−	+−	+−	−
15	1 "	+	+	+	−
16	1 "	+	+−	−	−
17	1 "	+−	−	−	−
18	1 "	+−	+	+−	−
19	1 "	−	−	−	−
20	1 "	++	+	+−	−
21	1 "	+	−	−	−
22	1 "	+	−	+−	−
23	1 "	+−	+−	−	−
24	1 "	++	+	+−	−
25	1 "	+−	−	+−	−
26	1 "	+	−	+−	−
Controls.					
0	2 units.	−	−	−	−
0	0	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

Although the one unit of complement used was a very small quantity (0.04 cc.) the possibility of the influence of the natural anti-human hemolysin in the complement against Group I and Group II cells could not be overlooked. The above experiment was repeated with complement from which the anti-human hemolysin had been previously absorbed. The complement was prepared in the usual way and was tested against all four groups of human cells. The natural hemolysin was then absorbed from it by treating it with Group II cells, at freezing temperature, for ½ hour. After absorption the complement was retested against all four group cell suspensions to verify the complete absorption of the hemolysin, in at least eight times the quantity used as one unit. Twenty-six sera were tested, as shown in Table VIII, the quantities used being the same as in the first experiment (Table VII).

The results obtained were similar to those shown in Table VII. With the natural hemolysin absorbed from the complement, hemolysis of some degree took place with practically all the sera and Group I cells, and with many of the sera and Group II and Group III cells, showing that the natural anti-human hemolysin present in the one unit of complement used in the first experiment was not entirely responsible for the hemolysis obtained with Group I and Group II cells.

The foregoing work has made it evident that, theoretically at least, the blood group to which the cell suspension belonged might play as important a part in the anti-human complement fixation reactions as the presence of natural anti-sheep amboceptor in human serum was supposed to play in the methods in which an anti-sheep hemolytic system was used.

Influence of an Excess of Amboceptor upon Complement Fixation Reactions in Which an Anti-Human Hemolytic System Is Used.

To determine the real practical importance of both the isolysins in human serum and the natural anti-human hemolysin in guinea pig serum, a series of complement fixation tests was done upon a number of sera which had given strongly positive, weakly positive, and doubtful reactions in the routine tests, all four group cells for each serum tested being used. Two units of complement were used, the

titer having been determined for use in the routine tests that day. The results are shown in Table IX.

Of the twenty-six sera tested there were but eleven that gave the same reacton with all four group cells, three being known negative

TABLE IX.

Complement Fixation Tests on Twenty-Six Human Sera against the Four Groups of Human Cells.

Serum No.	Reaction with a suspension of cells of.			
	Group I.	Group II.	Group III.	Group IV.
1	−	−	−	+−
2	−	+−	+−	+−
3	+	++	++	++
4	++	++	++	++
5	−	+−	+	++
6	−	+−	−	+−
7	+−	−	+−	+−
8	−	+−	+−	+−
9	++	++	++	++
10	++	++	++	++
11	++	++	++	++
12	+	+	+	++
13	++	++	++	++
14	+−	+	+	++
15	+	+	+	++
16	++	++	++	++
17	++	++	++	++
18	+	+	+	++
19	+−	+	+	+
20	−	−	−	−
21	+−	+	++	++
22	+−	++	+	++
23	+	++	++	++
24	++	++	++	++
25	−	−	−	−
26	−	−	−	−

++ indicates complete inhibition of hemolysis; +, partial inhibition of hemolysis; +−, slight inhibition of hemolysis; −, complete hemolysis.

Particular attention was paid to technique in these tests, all being run at the same time under identical conditions, the same reagents being used for all, and all steps being strictly controlled. Sera 20, 25, and 26 were known negative sera that were introduced as hemolytic and antigen controls.

sera introduced as controls. The fifteen sera showing variations, as a rule, gave the weakest reaction with Group I cells, but the results with Group II and Group III cells were sufficiently marked to show that the group of the cell suspension used plays an important part in the strength of the reaction obtained with the human hemolytic system.

TABLE X.

Complement Fixation Tests on Seventeen Human Sera against the Four Groups of Human Cells with Complement from Which the Natural Anti-Human Hemolysin Had Been Absorbed.

Serum No.	Reaction with a suspension of cells of.			
	Group I.	Group II.	Group III.	Group IV.
1	++	++	++	++
2	+−	+	+	++
3	++	++	++	++
4	+−	+	+	++
5	++	++	++	++
6	++	++	++	++
7	+	+	+	++
8	+−	+	+	+
9	++	++	++	++
10	−	−	−	−
11	−	+−	−	+−
12	−	−	−	+−
13	−	+−	−	+
14	−	+	+	+
15	+−	+	+−	+
16	+−	++	++	++
17	−	−	−	−

++ indicates complete inhibition of hemolysis; +, partial inhibition of hemolysis; +−, slight inhibition of hemolysis; −, complete hemolysis.

As the above tests had been done with the reagents and technique as ordinarily used in the routine complement fixation reactions, it was difficult to judge just how much of the variation in the reading was strictly due to the isolysins in the human sera and how much to the natural anti-human hemolysin present in the complement used. A second set of tests was run with various sera, all four groups of cell suspensions being used for each serum. The complement employed

had had the natural anti-human hemolysin absorbed from it. It was prepared in the usual way and tested against all four group cell suspensions. The natural hemolysin was then absorbed by treating the complement, at freezing temperature, with Group II human cells. After absorption the complement was retested against cell suspensions of all of the four groups to verify the complete absorption of the hemolysin. The unit of complement was determined by titrating against Group IV cells and two units were used in the tests. The same precautions as to technique, controls, etc., were observed here as with the first set of tests. Results were obtained as shown in Table X.

With the seventeen sera tested there were but seven that gave the same reaction with all four cell suspensions, five of these being strongly positive and two being known negative sera introduced as controls. With the ten remaining sera, weaker reactions were obtained with Group I, Group II, and Group III cells than were obtained with Group IV cells.

These results show that the isolysins in human serum are the important factor in the variations in the readings of the reaction obtained with the different group cell suspensions. The natural anti-human hemolysin in guinea pig serum may be a contributing factor to the resultant excess of amboceptor present when Group I or Group II cells are used, but it is of less importance, in the quantity ordinarily used in a two unit dose, than the human isolysins.

All the work so far points to the necessity, from the standpoint of accuracy, of using erythrocytes from a Group IV individual when preparing cell suspensions for work with a complement fixation reaction in which an anti-human hemolytic system is used. This is entirely compatible with our knowledge of the human blood groups, as Group IV cells are not acted upon by the isohemolysins in the serum of any of the human blood groups.

Human Blood Groups in Complement Fixation Reactions with an Anti-Sheep Hemolytic System.

Very early in the work reported above, the suggestion was made to the writer that the presence of natural anti-sheep hemolysin in human serum might bear some relation to the human blood groups. This could operate in two ways—either there might be certain of the

human blood groups whose sera contained hemolysin for all sheep cells, or, more possibly, there might be definite groups among sheep and it would perhaps be possible to select a sheep of a certain group whose cells might conceivably correspond to the human Group IV cells and contain no receptors for the hemolysins present in human serum. Such an observation as the latter, if it could be substantiated, would naturally be very important in complement fixation reactions with an anti-sheep hemolytic system.

TABLE XI.

Natural Anti-Sheep Hemolysin in Ten Human Sera Tested against Eleven Sheep Cell Suspensions.

Human serum (0.05 cc.).		Amount of complement (10 per cent).	Reaction with 0.25 cc. of a 5 per cent sheep cell suspension from.										
No.	Blood group.		Sheep A.	Sheep B.	Sheep C.	Sheep D.	Sheep E.	Sheep F.	Sheep G.	Sheep H.	Sheep I.	Sheep J.	Sheep K.
		cc.											
1	III	0.1	+	+	+−	+	−	++	−	+−	+−	+	+−
2	IV	0.1	++	++	++	++	++	++	++	++	++	++	++
3	III	0.1	−	+	−	+	+−	+	−	−	−	+	++
4	IV	0.1	++	++	++	++	++	++	++	++	++	++	++
5	III	0.1	++	++	++	++	++	+	++	++	+	++	++
6	IV	0.1	++	+−	+	++	++	++	++	+	++	++	++
7	II	0.1	++	+	+	++	+	++	+	+	+−	++	++
8	IV	0.1	+	+−	+	+	+	++	+	+	+	++	+
9	II	0.1	++	++	++	++	++	++	++	++	++	++	++
10	II	0.1	++	+	+	++	++	++	+	+	+	++	+

++, indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

The problem of the effect of various human sera upon different sheep cells was approached by testing ten human sera, the blood group to which each belonged being known, against eleven different sheep cell suspensions. The technique used was that employed in the usual quarter unit Wassermann test. Inactivated human serum was used in a dose of 0.05 cc., complement was a 10 per cent dilution of pooled guinea pig serum, and the cell suspensions employed were all 5 per cent with a dosage of 0.25 cc. The complement was titrated against

a sheep cell suspension obtained from our own sheep, whose cells are not included in the eleven tested as shown in Table XI. The complement was also tested for hemolytic properties in a two unit dose against all of the eleven sheep cell suspensions used. The results are given in Table XI.

TABLE XII.

Natural Anti-Sheep Hemolysin in Sixteen Human Sera Tested against Twelve Sheep Cell Suspensions.

Human serum (0.05 cc.).		Amount of complement (10 per cent).	Reaction with 0.25 cc. of a 5 per cent sheep cell suspension from.											
No.	Blood group.		Sheep L.	Sheep M.	Sheep N.	Sheep O.	Sheep P.	Sheep Q.	Sheep R.	Sheep S.	Sheep T.	Sheep U.	Sheep V.	Sheep W.
		cc.												
11	III	0.1	+−	+−	−	+−	−	+	−	−	+−	+−	+	+−
12	IV	0.1	++	++	+	++	−	++	+	++	++	++	++	+
13	III	0.1	+	+	+−	+	−	+	+−	+	+	−	+	+
14	IV	0.1	++	+	+	+	−	++	+−	+	++	+	++	+
15	III	0.1	++	++	+	++	−	++	+	++	+	++	+−	++
16	IV	0.1	++	−	++	++	++	+	+	++	++	−	+	++
17	II	0.1	+	+−	−	+	+	+	+−	+	+	+−	+	+
18	IV	0.1	+−	++	++	+	+	+	−	+	+	+−	+	+−
19	II	0.1	++	++	++	++	++	++	+	+	++	++	++	+−
20	II	0.1	++	++	+	++	++	++	+	+	++	+	++	+
21	IV	0.1	−	−	−	−	−	−	−	−	−	−	−	−
22	IV	0.1	−	−	−	−	−	+	−	−	−	−	+	−
23	IV	0.1	−	−	−	−	−	−	−	−	−	−	+	+−
24	II	0.1	+−	++	++	++	−	++	++	++	++	++	++	++
25	IV	0.1	+−	+−	++	+	−	−	−	−	++	−	++	+
26	IV	0.1	++	++	++	++	−	++	++	++	++	++	++	++

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

The results obtained were so variable and irregular that the experiment was repeated. Human serum from sixteen different individuals, the blood group of each of whom was known, was used against twelve entirely different sheep cell suspensions. The technique employed was identical with that used above, and the same precautions were taken in testing and titrating the complement. The results are given in Table XII.

In Tables XI and XII are given the results on twenty-six different human sera, comprised in the commonest blood groups, ten of the sera being tested against eleven sheep cell suspensions and sixteen of the sera against twelve different sheep cell suspensions. All efforts to corelate these results with the various blood groups of the sera tested were futile. There appeared to be no regularity with which the serum from any particular blood group acted upon the cell suspensions.

On the other hand, there was a marked variation in the degree of hemolysis obtained with many of the individual sera against the different sheep cells. With certain sera this would vary from no hemolysis with one sheep cell suspension to complete hemolysis with another cell suspension, and almost all the human sera showed some variation in their action against the various cell suspensions. This fact is undoubtedly of some significance, although as can be seen, among the twenty-three sheep cell suspensions tested there was no single sheep whose cells failed to contain receptors for the anti-sheep hemolysin present in at least two or more of the human sera.

Further investigation along this line is being continued and these results, while unsatisfactory, are reported in order that other workers may investigate this variation in the susceptibility of different sheep cells to the natural anti-sheep hemolysin present in human serum.

Natural Anti-Sheep Hemolysin in Guinea Pig Serum.

Another problem to be considered was the occasional presence of natural anti-sheep hemolysin in guinea pig serum used as complement. That this may be of considerable importance in occasional instances has been shown above. During one period in which it was encountered in France approximately 150 guinea pigs from one lot had to be bled in order to obtain sufficient non-hemolytic serum that could be pooled and used as complement in the routine work. It occurred sporadically at that particular laboratory over a long period of time. It seemed strange, however, that during this period many competent observers in various subsidiary laboratories in France failed to find this hemolysin at any time in their guinea pig serum. At the time this was ascribed to a probable difference in the guinea pigs being

used, differences in breeding and inbreeding, as well as the possible influence of environment and food all being considered.

The results reported here would indicate that these views were probably erroneous and that the trouble was possibly due to a certain type or group of sheep cells for which certain guinea pig serum contains hemolysin, rather than to any inherent difference in the individual guinea pigs. This explains the failure of certain observers to confirm our observation, the particular sheep cells that they used not being susceptible to the action of, or not containing receptors for the hemolysin present in guinea pig serum. At the laboratory in question three sheep were in use most of the time, and so far as the writer knows, no strict rule was in force relative to the rotation of the bleedings. The sheep that was easiest to catch and hold was usually the sheep that was bled the most often.

When the work on the natural anti-human hemolysin in guinea pig serum showed that this hemolysin was selective for Group I and Group II human cells, it suggested the possibility of the presence of a natural anti-sheep hemolysin in guinea pigs which would only be active against a certain type or group of sheep cells. A large number of guinea pigs was tested against many sheep cell suspensions on different occasions. The guinea pig serum was used in a dilution of 10 per cent in amounts as indicated in Table XIII; cell suspensions were 5 per cent with a dose of 0.25 cc. In order to eliminate the possibility of anticomplementary action by any of the sheep cells, a hemolytic control was run on each cell suspension, with 0.2 cc. of the 10 per cent guinea pig serum and one unit of an artificial anti-sheep amboceptor. Detailed tabulation of the results need not be given, but a sample protocol of the test of four guinea pigs against seven sheep cell suspensions is shown in Table XIII.

In addition to the tests described above, twelve guinea pig sera against a total of fourteen different sheep cell suspensions gave negative results, which would indicate that it is a relatively uncommon condition for the average sheep cells to contain receptors for the natural hemolysins present in guinea pig serum in the quantities ordinarily used in complement fixation work. Table XIII, however, shows graphically what would happen if the sheep used for preparing the cell suspension should correspond to Sheep L. These results

would also explain the fact that a natural anti-sheep hemolysin was encountered with such frequency at one laboratory in France, while other observers failed to find this hemolysin in guinea pigs in use at other laboratories. The possibility of its occurrence, however slight, should be considered when selecting a sheep for complement fixation work.

TABLE XIII.

Natural Anti-Sheep Hemolysin in Guinea Pig Serum Tested against Seven Sheep Cell Suspensions.

Guinea pig No.	Amount of 10 per cent dilution of guinea pig serum.	Reaction with 0.25 cc. of a 5 per cent sheep cell suspension from.						
		Sheep L.	Sheep M.	Sheep N.	Sheep P.	Sheep R.	Sheep S.	Sheep W.
	cc.							
54	0.1	−	−	−	−	−	−	−
	0.2	+−	−	−	−	−	−	−
	0.3	+	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−
55	0.1	−	−	−	−	−	−	−
	0.2	+−	−	−	−	−	−	−
	0.3	+	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−
56	0.1	+	−	−	−	−	−	−
	0.2	++	−	−	−	−	−	−
	0.3	++	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−
57	0.1	+−	−	−	−	−	−	−
	0.2	+−	−	−	−	−	−	−
	0.3	+	−	−	−	−	−	−
	0.4	++	−	−	−	−	−	−

++ indicates complete hemolysis; +, marked hemolysis; +−, slight hemolysis; −, no hemolysis.

SUMMARY.

1. The presence of a natural anti-human hemolysin, only active against Group I and Group II cells, was demonstrated in over 50 guinea pigs, obtained from different sections of the surrounding country. It was shown that, in a majority of the sera tested, this

hemolysin would be a contributing factor to an excess of amboceptor in the reaction, in the quantities of complement used in an anti-human complement fixation reaction. The hemolysin is specific, in as far as it can be absorbed with either Group I or Group II cells, but cannot be absorbed with Group III or Group IV cells.

2. To determine the effect of the isohemolysins in human serum, twenty sera were tested against cell suspensions of the four human groups. Tests were also run with twenty-six human sera against all four group cell suspensions, with complement that had the natural anti-human hemolysin absorbed from it. In both experiments various degrees of hemolysis were obtained with all but two sera against cell suspensions from Groups I, II, and III, but no hemolysis was obtained with any serum against a Group IV cell suspension.

3. The importance of the human isohemolysins and the natural anti-human hemolysin in complement was demonstrated by performing complement fixation tests upon twenty-six human sera, cell suspensions of all four human groups for each serum tested being used. A marked variation in the reaction occurred with a large percentage of the sera tested. This variation in a single serum might be as great as a negative reading with Group I cells to a double plus reading with Group IV cells.

4. That the isohemolysins, alone, in human serum are of importance in the reaction was shown by running complement fixation tests upon seventeen human sera with the four group cell suspensions and complement from which the natural anti-human hemolysin had been absorbed. Results showed a much weaker reaction, in a large number of the sera, with Group I, Group II, and Group III cells than with Group IV cells.

5. Experiments to determine a relation between the human blood groups and natural anti-sheep hemolysin present in human serum gave negative results. It was shown, however, with twenty-six human sera tested against twenty-three different sheep cell suspensions that there is a wide variation in the activity of the natural anti-sheep amboceptor present in any human serum when it is tested against different sheep cell suspensions. A human serum capable of completely hemolyzing the cells of one sheep may fail to give any hemolysis whatever when tested against another sheep cell suspension.

6. Tests were made for natural anti-sheep hemolysin in guinea pig serum. The serum from sixteen different guinea pigs was tested against a total of twenty-one sheep cell suspensions. A natural anti-sheep hemolysin was present in all the guinea pigs tested against one of the sheep cell suspensions. The same guinea pigs tested against the other sheep cell suspensions failed to show any hemolysis.

CONCLUSIONS.

1. In the Wassermann reaction it should be taken into account that in addition to any complement-fixing bodies which may or may not be present, each serum tested belongs to a definite blood group and each human cell suspension used belongs to a definite blood group. These factors have never been sufficiently realized and must be taken into consideration if possible sources of error are to be excluded.

2. In addition to the isohemolysins in human serum, an additional source of error, when human cell suspensions are made up at random, is introduced by the presence of a natural anti-human hemolysin in guinea pig serum selective for Group I and Group II human cells.

3. Erythrocytes from a Group IV individual (Moss' classification) are never acted upon by the isohemolysins present in human serum nor are they acted upon by the natural anti-human hemolysin present in guinea pig serum.

4. Erythrocytes should always be obtained from a Group IV individual when preparing cell suspensions for complement fixation reactions in which an anti-human hemolytic system is used.

5. An unexplained variation in the activity of the natural anti-sheep hemolysin in human serum, when tested against different sheep cell suspensions, would seem to be worthy of further investigation.

6. The presence of a natural anti-sheep hemolysin in guinea pig serum used as complement, apparently only active against a certain type or group of sheep cells, is a factor to be considered when selecting a sheep for complement fixation work.

I wish to express my appreciation to Colonel F. F. Russell, Colonel C. F. Craig, and Major H. J. Nichols for helpful criticisms during the progress of this work.

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EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

III. Nephrotoxins: Their Specificity as Demonstrated by the Method of Selective Absorption.

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Plates 5 to 7.

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The present study was undertaken as a continuation of work begun in this laboratory on the production of antisera for infections of unknown cause. It was found[1] that the infected tissues of animals might serve in some cases as an antigen for the production of immune sera, though with such antigens the sera inevitably contained antibodies injurious to the tissues of the animal. Experiments showed, however, that the sera can be largely deprived of these latter by incubation with successive portions of red cells without impairment of their protective value. Whether there are substances injurious to special organs which cannot so be taken out has remained to be determined. The problem is closely connected with that of the specificity of cytotoxins.

For most organs truly specific cytotoxins have not as yet been produced, despite many attempts. They have been elicited, though, for certain organs more or less isolated in the body. Zinsser sums up as follows on the subject:[2]

"Recent critical studies . . . . have revealed . . . . that the specificity of a serum produced with the tissues of one organ is not strictly limited to this organ alone, and that the serum may injure other organs as well. It is true, indeed, that there are certain cells and tissues in the body such as spermatozoa, the tissues of the testicles, the ovary, the lens of the eye, and possibly

[1] Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

[2] Zinsser, H., Infection and resistance, New York, 1914, 92.

the placenta, which have chemical characteristics so well defined and individual that the cytotoxic sera induced by them have definite organ specificity. The same to a more limited extent seems true of kidney substance (Pearce). In most cases, however, in which originally a specific cytotoxin was claimed, it has been possible to show subsequently that the apparently selective injury was not due to organ specificity alone but to the fact that the injection of tissue macerates, even when sufficiently freed from blood, induced the formation of considerable amounts of hemagglutinins and hemolysins."

In the light of such facts our problem resolves itself into a comparatively simple one: to determine whether one of the so called specific cytotoxins can be removed from a serum by exhausting the latter with red cells.

In selecting an organ for the study of "specific" cytotoxic sera the kidney seemed best adapted, because injuries to it can be determined by functional as well as histological methods. Pearce[3] has brought forward evidence that a serum can be produced which is, as he says, at last "special" in its action on the kidney. He leaves in doubt whether it can be termed specific in the strict sense, for despite the use of blood-free kidneys as antigen, his sera invariably contained hemolysins and hemagglutinins which might possibly account for their damaging effect on the kidney.

We have undertaken, first, to obtain a serum such as Pearce describes and observe its effects on the kidney; second, to determine whether the principle injurious to the kidney can be removed from this serum by absorption with red blood cells or with kidney tissue; and, third, to compare the efficacy of serum exhausted with red cells and that similarly treated with kidney tissue.

Production of Antikidney Serum.

Pearce's method was followed.[3]

Large dogs were etherized and under special precautions for asepsis their kidneys were removed and placed in glass boxes. The neighboring portion of the aorta was left attached to the kidneys in order to facilitate the passage of a cannula into the renal artery; and by prolonged washing with sterile salt solution the kidneys were practically freed of blood. The capsules were then stripped,

[3] Pearce, R. M., *J. Med. Research*, 1904, xii, 1.

the kidneys weighed and put through a meat grinder. The macerated tissue was pushed through a tea strainer of fine mesh with the aid of a pestle, small quantities of salt solution being added from time to time to facilitate its passage. The entire procedure was carried on under a glass plate surrounded by a canopy of sterile muslin which effectively protected the material from contamination.

The resulting heavy suspension in salt solution passed readily through a needle of medium sized bore. The material was usually injected into animals within 48 hours of its preparation, though occasionally it was kept in a frozen condition for some days prior to use. A number of rabbits received three to five intraperitoneal injections, each of from 4 to 8 gm. of kidney tissue, at intervals of 7 days, and they were bled on the 10th day after the last one. The sera were inactivated (56°C. for ½ hour) prior to use and their hemolytic and hemagglutinating titers tested, and cultures taken.

The sera without exception possessed moderately strong hemagglutinins and weak hemolysins for dog cells. Tests were made in the usual manner with graded dilutions in salt solution. Equal portions of the serum dilutions and of a 5 per cent suspension of thrice washed dog cells were used in the agglutination tests, and for hemolysis guinea pig complement in a dilution of 1 to 10 was added.

Precipitins were tested for but when found were negligible in amount.

Effects of Antikidney Serum.

Nine dogs were given a single intravenous injection of inactivated antikidney serum. The dose was 1 to 2 cc. per kilo of body weight. In most cases extremely marked renal changes resulted, differing considerably from those described by Pearce.

Prior to injection the general condition as well as the urine of the animals was carefully followed for a period of at least 2 weeks. Many supposedly normal dogs show albumin and casts in the urine without evident cause, and much delay was often experienced in finding animals suitable for our purpose. Within 24 to 48 hours after injection the animals appeared rather lethargic and gave the general impression of being sick. This period of depression passed off completely, or persisted, according to the potency of the serum and corresponding in a general way to the damage sustained by the kidney. The changes in the urine were marked. Severe albuminuria rapidly developed, with many casts, mostly granular at first but later many of them hyaline and occasionally waxy. This always occurred within 48 hours after the injection of the serum and persisted for the few days before the dog was killed. A trace of blood was sometimes noted.

With the exception of one dog (Dog C), which was observed for 4 months, all of the dogs were chloroformed and autopsied 10 to 14 days after injection. The kidneys were removed just prior to death or immediately after it. The viscera were examined, but no lesions were ever found that could be attributed to the serum. On two occasions more than 750 cc. of clear straw-colored fluid were present in the peritoneal cavity. No general edema was ever observed.

The kidneys showed pathological changes practically always, often of marked degree. In typical cases the organs were enlarged, and on stripping the capsule the kidney surface was everywhere mottled with bright red dots, from 0.1 to 1 mm. in diameter (Fig. 1, *A*). On section these were found to be the surface indices of hemorrhages into the cortex, the most characteristic gross finding. The hemorrhages were in general wedge-shaped, with the base of the wedge at the kidney surface. The glomeruli were indistinct and apparently had no relation to the hemorrhages. The cortex was swollen and opaque, and the pale medulla occasionally showed traces of hemorrhage extending from the cortex.

Histologically glomerular lesions, not the hemorrhages, were the most marked as well as constant finding. They occurred without hemorrhages when the serum was weak. In well marked instances there was an extensive necrosis in the glomerular tufts followed by obliterative hyaline changes (Figs. 2 and 3). A large proportion of the glomeruli were thus affected, though in even the most pronounced instances a sufficient number remained for kidney function. Accompanying the early cell changes was a plugging of the glomerular capillaries with fibrin and often a fibrinous exudation into the capsular space (Fig. 4). Desquamation and proliferation of the cells of the loops with occasional mitotic figures were observed. The end-result was often a complete obliteration of the glomerulus.

According to Pearce the most striking changes occur in the tubules. In our experiments lesions there were usually not pronounced, though sometimes marked cloudy swelling and desquamation were observed.

Next to the glomerular changes already described the most frequent findings were casts and hemorrhage into the tubules (Figs. 2 and 4). When the serum was strong small groups of a dozen to twenty tubules, usually in the neighborhood of a glomerulus, had their lumina filled

with blood cells (Fig. 5). These were the punctate hemorrhages so prominent in the gross specimen (Fig. 1).

There was also observable sometimes a considerable round cell infiltration between the tubules, especially in the neighborhood of the glomeruli. Some of the destroyed glomeruli showed a very heavy infiltration with lymphocytes and polymorphonuclear leucocytes (Fig. 6).

As a control two dogs were given large doses of normal rabbit serum. The results were negative as regards changes in the kidneys.

Effect of Antikidney Serum Exhausted with Red Cells.

The sera which caused the lesions described above contained, as already stated, hemolysins and hemagglutinins, though these were never strong. Steps were now taken to determine whether the action of the serum was due to them. Pearce performed a single experiment of the sort, submitting serum to contact with washed dog red cells for 1 hour at 0°C., and he found it thereafter "only faintly hemolytic." Kidney lesions were still produced. He records no observations, however, on hemagglutinins in the serum, which are often stronger and persist longer than hemolysins. In view of their ability to cause liver necroses,[3] such antibodies might well have been responsible for the lesions occurring in the kidneys.

By repeated absorptions with washed dog red blood cells, the technique of which has been previously described in detail,[1] our sera were completely deprived of hemolysins and hemagglutinins.

The results of the injection of dogs with serum thus treated are contrasted in Table I with findings in control animals receiving injections of corresponding doses of the same serum, untreated.

As the table shows, the exhaustion of the serum with red cells does not affect its ability to cause urinary changes. The albuminuria and cast formation were slightly less marked than in the controls. On the other hand, there were to the naked eye striking gross differences in the kidneys of the two series. It has been said that the untreated serum causes punctate hemorrhages in the renal cortex, often so numerous as to mottle diffusely the kidney surface. They are almost, or completely, lacking in animals given exhausted serum

TABLE I.

Effects of Nephrotoxic Sera, Untreated and Exhausted with Red Cells.

Experiment No.	Dog.	Serum No.	Treatment.	Serum titer. Action disappears at dilution of.	Dose per kilo of animal.	Effect on urine.		Killed after.	Condition of kidneys.
						Albumin.	Casts.		
					cc.			*days*	
1	A		Untreated.	1/256 lysis. 1/128 agglutination.	1.55	+−	++	8	Slight gross changes; characteristic glomerular lesions and microscopic hemorrhages. Casts.
	Aa	1*	11 cc. of serum underwent four absorptions with 1 cc. of red blood cells in all.	No lysins or agglutinins.	1.59	−	+	8	A few casts in tubules. Marked epithelial lesions.
2	B		Untreated.	1/256 lysis. 1/128 agglutination.	1.33	−	+	8	Marked epithelial lesions. Casts.
	Ba	2*	13.5 cc. of serum underwent four absorptions with 16 cc. of red blood cells.	No lysins or agglutinins.	1.72	+	−	8	Moderate epithelial lesions.
3	C		Untreated.	1/4 lysis. 1/1,024 agglutination.	1.65	+-+	+++	119	Few fibrotic glomeruli. No increase of interstitial tissue.
	Ca	3	17 cc. of serum underwent five absorptions with 25 cc. of red blood cells.	No lysins or agglutinins.	1.60	+++	+++	19	Severe glomerular lesions and hemorrhages. Many casts.

4	D	5	Untreated.	1/10 lysis. 1/30 agglutination.	0.60	++	++	9	Moderate glomerular lesions and hemorrhages. Many casts.
	Da		37 cc. of serum underwent five absorptions with 17 cc. of red blood cells.	No lysins or agglutinins.	1.36	++	+	9	Changes definite, but less than in the control.
5	E	5	Untreated.	As in Experiment 4.	1.85	++	++	10	Marked glomerular lesions. Hemorrhages and casts.
	Ea		Absorbed as in Experiment 4.	As in Experiment 4.	1.86	++	+	10	As in control, but slightly less marked.
6	F	6	Untreated.	1/4 lysis. 1/512 agglutination.	1.66	++	++	11	Numerous hemorrhages and glomerular lesions. Many casts.
	Fa		22 cc. of serum underwent seven absorptions with 22 cc. of red blood cells.	No lysins or agglutinins.	1.85	++	++	11	As in control.
7	G	7	Untreated.	1/128 lysis. 1/128 agglutination.	1.29	+++	++	10	Moderate changes of characteristic sort.
	Ga		15 cc. of serum underwent eleven absorptions with 34.5 cc. of red blood cells.	No lysins or agglutinins.	1.00	+++	++	10	As in control, but slightly fewer glomerular lesions.
	H		Untreated.	1/128 lysis. 1/128 agglutination.	0.65	++	++	11	Moderate changes of characteristic sort.
8	I	4	"	1/128 lysis. 1/16 agglutination.	1.65	+++	+++	22	Characteristic but moderate lesions.

* Sera 1 and 2 were kept 3 months before their absorption and the animal tests, which may explain the mildness of their effects.

(Fig. 1, *B*). To judge from the gross appearance one would suppose that exhaustion had entirely deprived the serum of its ability to cause renal lesions. Microscopically, however, it is seen that all of the lesions previously described are present save the hemorrhages. The glomerular and tubular lesions are in some instances less marked than in the controls, but in other cases they are equally well defined.

Type Experiment 1. Action of Nephrotoxic Serum. (*a*) *Untreated;* (*b*) *Absorbed with Red Cells.*—(*a*) Dog F, male, weight 6 kilos, after 2 weeks observation, during which time the urine was frequently examined and found to be free of casts and albumin, was given, on Mar. 26, 1918, 10 cc. of Serum 6 intravenously. The dog behaved normally after the injection. (This serum was prepared by injecting Rabbits 1 and 2 with blood-free dog kidney suspension. On Oct. 1, 1917, the suspension contained 4 gm. of kidney tissue; Oct. 8, 6 gm.; Oct. 15, 8 gm.; Oct. 22, 5 gm. Oct. 30. The rabbits were bled to death and the serum preserved in the ice box. On Mar. 22 the two sera were pooled and the titer was determined. Hemolysis disappeared at a dilution of 1 to 4 and agglutination at 1 in 512. Inactivation was done at 56°C. for ½ hour.)

Mar. 27. Urine very dark amber, alkaline, cloudy. Dense ring of albumin with nitric acid test. Many epithelial cells, a few red blood cells and leucocytes. No casts. Guaiac test positive. Mar. 28. Urine shows a great deal of albumin. Guaiac test positive. Occasional granular casts. Mar. 30. Same findings. Apr. 1. Albumin present. Guaiac test negative. Very many hyaline and granular casts. Apr. 3, 5, and 6 showed the same findings.

On Apr. 6 the dog was etherized and an autopsy performed, with negative findings in all the organs except the kidneys, which were swollen and congested. On stripping the capsule innumerable fine red dots up to 1 mm. in diameter were revealed on the surface of the kidney (Fig. 1, *A*). On section the kidneys were swollen and opaque, the cortex showing a definite widening.

Histological examination showed innumerable punctate cortical hemorrhages into the tubules, filling the lumina of scattered groups. There were a few interstitial hemorrhages. The majority of the glomeruli showed marked changes. Many of the cells in the tufts were necrotic, and numerous coils were plugged with fibrin. Mitoses were not infrequent. There was some interstitial round cell infiltration here and there between the tubules, especially in the neighborhood of the glomeruli. Many casts were seen in the tubules, but only slight changes in their epithelium.

(*b*) Dog Fa; weight 5.25 kilos. Observed 2 weeks, during which the urine was free from albumin and casts. The animal was given intravenously, on Mar. 26, 1918, 9.8 cc. of Serum 6, which had been absorbed six times with washed dog red blood cells. (The total amount of serum submitted to absorption was 22 cc. 3 cc. of washed dog red cells were added to it, the tube inverted several times and incubated at 37°C. for 1 hour. Dense agglutination resulted. After centrifu-

gation the serum was pipetted into another tube, another 3 cc. of washed cells were added, and incubation was repeated. In all, seven such absorptions were carried out on this serum, after which it was tested for hemolysins and agglutinins and found to possess none. Cultures taken prior to its use for injection proved that it was sterile.)

Mar. 27. Urine showed a trace of albumin, no casts. Guaiac test positive. Mar. 28. Same findings. Occasional granular casts. Mar. 30. Large amount of albumin, many granular casts, no blood. Apr. 3, 5, and 6. Same findings. Apr. 6. The dog was etherized and all organs found to be normal except the kidneys, which were large and pale. The capsules were stripped and revealed a few pin-point hemorrhages, probably one for every fifty seen in the case of Dog F (Fig. 1, *B*).

Histological examination revealed changes almost identical with those observed in the case of Dog F, except that there were fewer hemorrhages (Fig. 2).

Effect of Ordinary Hemolytic-Hemagglutinative Serum.

In view of the fact that hemolysins have far more effect *in vivo* than *in vitro*,[4] it seemed necessary to test the effect on the kidney of an ordinary hemolytic and hemagglutinating serum produced by the injection of dog red cells. For it might be contended, as explaining the injurious effect on the kidney of our exhausted serum, that such serum still contained traces of hemolysin and hemagglutinin, which, while not demonstrable *in vitro*, were active *in vivo*. Rabbits were immunized therefore against washed dog red cells and an anti-dog serum of high agglutinin and hemolytic titer was produced. This was inactivated and injected intravenously into dogs, a part of it after absorption with dog red cells.

Type Experiment 2. Effect of Hemolytic and Hemagglutinating Serum. (a) Untreated; (b) Absorbed with Red Cells.—The serum used was prepared by injecting rabbits intraperitoneally with a 20 per cent suspension of thrice washed dog cells in salt solution. Five injections were made at 7 day intervals, and the rabbits were bled on the 8th day following the last injection. The resulting sera were pooled and found to be hemolytic in dilutions up to 1 in 256 and agglutinative in dilutions up to 1 in 1,025. A portion of the pooled serum was subjected to eleven absorptions with dog red cells and again tested, with the result that no hemolysins or agglutinins were found. In detail the method of absorption was as follows:

[4] Muir, R., and M'Nee, J. W., *J. Path. and Bacteriol.*, 1911–12, xvi, 410.

Absorption.	Amount of serum.	Dog red cells.	Incubation.
	cc.	*cc.*	*min.*
1	20	3	30
2		8	30
3		3.5	40
4–11		4 each.	60–120

(*a*) Dog R, weight 5.5 kilos, was given, on May 6, 1918, 2 cc. of the untreated portion of the serum. May 8. The urine, previously normal, is now smoky in appearance, containing a trace of albumin and much hemoglobin but no casts. May 10. Urine dark red; faint trace of albumin; many granular casts containing brownish pigment. Tests for blood and bile positive. May 14. Urine dark amber; albumin negative; no casts. Bile pigment and blood present. May 17. Urine amber, no albumin, hemoglobin, or casts. May 18. Dog etherized and kidneys removed. Histological examination showed much brown, granular pigment in the spleen and liver. The kidneys were entirely normal except for widespread, fairly abundant, light brown pigment granules in the cells of the proximal convoluted tubules.

(*b*) Dog S, weight 5.25 kilos, was given intravenously on May 2, 1918, 12 cc. of the absorbed portion of the serum. Immediately after the injection dog vomited, was prostrated, and passed feces. Complete recovery took place in 1 hour. The urine was examined on May 3, 4, 6, 8, 10, and 14, but on none of these occasions were any abnormal constituents found. On May 14 the dog was etherized. Autopsy showed all organs normal. On histological examination the kidneys were normal.

It will be seen that the unabsorbed serum produced no important anatomical changes in the kidney despite the marked blood destruction it caused. Pearce, using such a serum, reports a fatty condition limited almost entirely to the loops of Henle. Otherwise the urinary and kidney findings which he reports in animals that survived a few days were the same as here reported. He did not try absorption, which, as we found, renders the serum innocuous to the blood as well as the kidneys. Altogether the facts warrant the conclusion that the changes produced by an antikidney serum exhausted with red cells cannot be attributed to persisting hemolysins or agglutinins. It is interesting that the most striking gross lesion caused by the antikidney serum, namely the punctate cortical hemorrhages, is not produced by an anti-red-cell-serum, although absorption with red cells deprives the antikidney serum of its ability to produce such a lesion.

Effects of the Exhaustion of the Serum with Kidney Tissue.

Can the injurious principle in the antikidney serum be removed by absorption with kidney tissue? This point was now investigated.

For the purpose of proper absorption with kidney tissue it was obviously necessary that the latter should be in as finely divided a state as possible, so as nearly to resemble in amount of absorbing surface a red cell suspension. Coarse fragments of tissue would offer relatively little surface and might be expected to yield poor results.

To obtain a fine suspension the tissues of washed kidneys of dogs were ground in the way already described, taken up in normal salt solution, and shaken with broken glass for 1 hour. The glass and larger tissue fragments were then removed by slow centrifugation, leaving a dense, milky suspension of finely divided material. On microscopic examination many free kidney cells were found in this fluid, which was now sedimented at high speed, and the sediment repeatedly washed by centrifugation until the supernatant fluid came away perfectly clear. The sediment was found to consist of parenchymal and other cells and fragments of them. For the purpose of absorption it was measured in bulk and used in suspension in the same manner as blood cells. It went into suspension readily.

Type Experiment 3 (Table II). Inactivated Antikidney Serum X Was Divided into three Portions: (a) Untreated; (b) Absorbed with Red Blood Cells; (c) Absorbed with Different Amounts of Kidney Tissue.—The untreated serum weakly agglutinated dog red cells in a dilution of 1 to 32 and hemolyzed them completely in a dilution of 1 to 4.

(*a*) *Results with Untreated Serum.*—Dog U, weight 7 kilos, with a urine negative for albumin and casts, received 1.35 cc. per kilo of the untreated serum on June 11, 1918. No symptoms following the injection. The urine on June 14, 19, 21, and 22 showed a heavy cloud of albumin and many hyaline and granular casts.

On June 22 the dog was etherized and an immediate autopsy performed. All organs were negative except the kidneys, which were swollen and congested. A few punctate hemorrhages were seen on the surface.

Histological Examination.—All organs negative except the kidneys, which showed characteristic changes. Many of the glomeruli were greatly damaged, though not a majority. All showed increase of cell nuclei, and the loops were often difficult to distinguish and appeared collapsed, apparently as a result of small occluding fibrin masses. There were some glomerular adhesions. The proximal convoluted tubules were definitely swollen, with much albuminous debris in the lumina, as well as many casts. The lumina of the descending portion of the loop of Henle were filled with red cells in places. There were small, scattered areas of edema of the interstitial tissues. The liver and spleen showed nothing abnormal.

(*b*) *Results with Serum Absorbed with Red Cells.*—Dog V, weight 8.5 kilos, received intravenously, on June 11, 1918, 1.28 cc. per kilo of Serum X, absorbed with dog red blood cells. The following was the method of absorption.

Absorption.	Amount of serum.	Dog red cells.	Incubation.
	cc.	*cc.*	*min.*
1	22	6	50
2		3.5	60
3–4		3 each.	60 and 90

After the absorptions there were no demonstrable hemolysins or agglutinins in this serum.) No symptoms. Urine negative.

June 13. Urine negative. June 14. Albumin, dense ring. No casts or blood. June 19 and 21. Urine showed much albumin, few granular and hyaline casts. On June 21 the dog was etherized. All organs were found negative with the exception of the kidneys, which were congested and swollen; no surface hemorrhages.

Histological Examination.—All organs negative with the exception of the kidneys, which showed a few interstitial hemorrhages. The usual glomerular changes were present. Few casts seen in the tubules. Findings like those of Dog U but not quite so pronounced.

Dog W, weight 4 kilos, received intravenously, on July 13, 1918, 4.4 cc. of Serum X, absorbed with red cells as above. July 15. Urine contained much albumin, occasional granular casts, no blood. July 17, 20, 22, and 24. The urine contained much albumin, many granular casts, no blood. Dog etherized and kidneys removed on July 24. Autopsy showed all organs normal with the exception of the kidneys, which were large and pale, with a very few punctate hemorrhages on their surface.

Histological Examination.—The general picture was the same as that of Dog U.

(*c*) *Results with Serum Absorbed with Kidney Cells.*—Dog X, weight 6.25 kilos, received, on June 11, 1918, 8 cc. of Serum X, absorbed three times with kidney cells in exactly the same proportion and for the same period employed with red cells as above stated. No symptoms. Urine examined June 13, 14, 19, 21, and 22. With the exception of June 14, when a very slight trace of albumin was observed, it was negative throughout. Etherization and autopsy on June 22. All organs normal by gross and histological examination.

Dog Y, weight 5.75 kilos, received, July 13, 1918, 8 cc. of Serum X, which had been absorbed three times with washed kidney cells of dogs as just described. No symptoms. Urine showed slight traces of albumin and occasional granular casts on July 15 and 17. On July 20, 22, and 24 it was negative. July 24. The dog was etherized and autopsied. Kidneys small, slightly congested, otherwise normal. All other organs normal.

Histological Examination.—Negative.

Dog Z, weight 4.50 kilos, received, on June 27, 1918, 2.2 cc. per kilo of Serum X, absorbed with kidney tissue as described. There followed a severe reaction, with vomiting, rapid respiration, and passage of feces and urine. The general picture suggested an anaphylactic crisis. After 20 minutes there was a gradual cessation of these symptoms, with complete recovery.

July 1. General condition of dog good. Urine showed albumin and a few granular casts but no blood. July 3. Urine about the same. July 5. Many casts.

TABLE II.

Relative Effects of Untreated Antikidney Serum and Serum Exhausted with Red Cells and with Kidney Cells (Type Experiment 3).

Dog.	Serum X.	Total bulk of sediment used for exhaustion per cc. of serum.	Dose of serum per kilo of animal.	Urine changes.	Kidney changes.
		cc.	*cc.*		
U	Untreated.		1.35	++	Moderate lesions in epithelium and glomeruli.
V	Four absorptions with red blood cells. Total incubation 5 hrs.	0.70	1.28	++	Slight glomerular lesions; moderate epithelial.
W	" "	0.70	1.10	++	Moderate glomerular lesions; no hemorrhages. Many casts.
Q	" "	0.70	2.17	++	Severe glomerular lesions; epithelial injury. Casts.
X	Three absorptions with kidney cells. Incubation 4 hrs.	0.50	1.26	0	Occasional cast. Otherwise negative.
Y	" "	0.68	1.39	0	Occasional cast.
Z	" "	1.00	2.2	+	Rare glomerular lesion. Few casts.

July 8. Only a trace of albumin, few casts. Dog etherized and autopsied. General examination negative. Kidneys showed no gross changes except a few hemorrhages, very few compared with control.

Histological Examination.—Occasional glomerular changes of the sort already described, but on the whole most of the glomeruli were in excellent condition. There were not infrequent characteristic hemorrhages into groups of tubules, as well as interstitial hemorrhages. Tubules generally normal, with the exception of a few casts in lumina. Liver and spleen normal.

The results of the experiment are summarized in Table II.

DISCUSSION.

Pearce's conclusion that an anti-dog rabbit serum could be produced which has a special action on the kidney has been confirmed. He, however, left the question of the true specificity of such a serum in doubt, and we must now consider whether our experiments throw any light on the subject.

An element in antikidney serum causing a very striking lesion, (abundant punctate cortical hemorrhages) is removed from the serum by the exhaustion of the latter with red cells, although an ordinary anti-red-cell-serum fails to produce such lesions. But the principle most injurious to the kidney cannot be so removed, even when the number of absorptions and the total bulk of red cells are very large. Moreover, a serum of high titer obtained by immunization with washed red cells fails to produce kidney lesions in any way resembling those of the nephrotoxic serum, which is further evidence that the injurious principle of the latter is not an hemagglutinin or hemolysin. The absorption of the antikidney serum with kidney tissue removes the injurious antibodies, even when the amount of tissue employed, and presumably the absorbing surface, are much less than were used in similar absorptions with red blood cells. Illustrations of the point are to be found in Table II.

The criticism may be made that the great absorptive power of the kidney tissue as thus demonstrated need not be the consequence of specificity but may be related to physical conditions that obtain in the emulsion, which make its absorptive power greater than that of a red cell emulsion. The experiments of certain observers on the absorption of antibodies with substances which obviously have no specific relation to them, kaolin, for instance, might here be cited. This criticism, however, is primarily directed not at the specificity of the nephrotoxin, with which we are alone concerned, but at the basic theory of the specificity of antibodies in general.

The effects of the nephrotoxic serum are exerted on the glomeruli and to a somewhat less degree on the tubules. It may be asked whether a vascular lesion will not account for all of the results. The glomerular changes are not improbably secondary to an occlusion of the coils following injury to the vascular endothelium. Our ani-

mals were autopsied too late to furnish conclusive evidence on the point. But whether or not the primary lesion is vascular, we are certain at least that the kidneys alone are affected. Were this the result of a non-specific endotheliolysin other organs should serve equally as well as the kidney for an antigen because of their content in endothelial cells. Pearce, however, has shown that a serum produced by the use of liver tissue as antigen has no effect on the kidneys.

As has been stated, our investigation was begun with a view to determining whether an antiserum produced as a result of the injection of tissues of an infected organ can be freed of injurious tissue antibodies by its exhaustion with red cells. As far as the kidney is concerned the question has been answered in the negative. A serum produced by immunization with kidney tissue should be absorbed with material from this organ. When that has been done it is no longer injurious to the kidney. Whether these principles are of general application may be doubted, for it is admitted that the kidney constitutes a special case. Notwithstanding numerous attempts by various investigators, sera specific for the spleen, pancreas, and liver have never been conclusively demonstrated. To obviate all possibility of injury from specific antibodies, however, a serum produced by the use of a tissue antigen should be exhausted with tissue of an identical sort prior to its introduction into the animal body.

SUMMARY.

As Pearce has shown, a serum highly injurious to the kidney of dogs can be produced by the immunization of rabbits with washed renal tissue of the dog. The histological findings are striking and characteristic, the most noteworthy being a glomerular lesion of special type. The renal changes differ much from those Pearce described.

The injury to the kidney is not to be explained by hemolytic and hemagglutinative elements in the serum. The complete removal of such antibodies by exhaustion of the serum with successive portions of red cells fails to lessen materially its ability to cause kidney lesions. Furthermore, an ordinary hemolytic and hemagglutinative serum produced by the use of washed red cells as antigen fails to cause similar lesions.

The distinctive, injurious principle of antikidney serum can be removed and the latter rendered innocuous by absorption with kidney tissue. To all practical intents and purposes it would seem that nephrotoxic serum of the sort here described is specific.

If infected tissue is to be utilized as an antigen for the production of therapeutic antisera the latter must in some instances be exhausted with tissue of the same sort prior to introduction into the animal body.

EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Photograph of the kidneys of Dogs F and Fa, Type Experiment 1, Table I. (*A*) The first mentioned animal received untreated antikidney rabbit serum, and its kidney shows innumerable cortical hemorrhages. (*B*) The other received an even larger amount of the same serum, from which the hemolysins and hemagglutinins had been removed by repeated absorptions with dog red cells. Though the kidney shows almost no hemorrhages, it was found microscopically to be badly damaged (Fig. 2).

FIG. 2. Section of the kidney pictured in Fig. 1 of Dog Fa, Type Experiment 1. Three glomeruli are seen, all of which show necroses of varying size. The tubules are dilated and filled with hyaline casts. There is beginning round cell infiltration in the neighborhood of the glomeruli. Bausch and Lomb, obj. $\frac{2}{3}$, oc. 1.

PLATE 6.

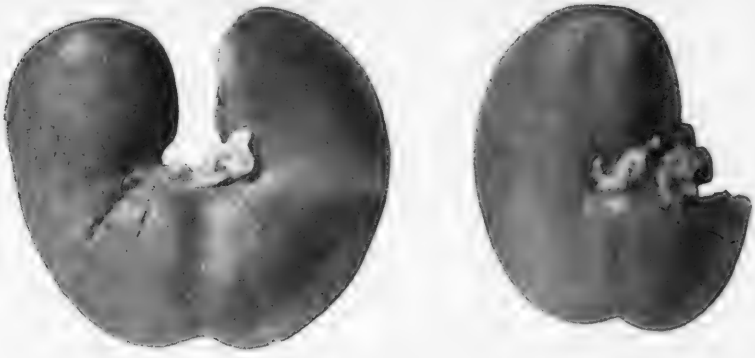
FIG. 3. Section from Dog Q, Type Experiment 3, which received antikidney serum absorbed with red cells. High power view of a necrotic glomerulus. Half of the glomerulus is entirely destroyed, and the other half severely compressed by the necrotic mass There is a beginning invasion of the dead material by proliferating capsular cells. Bausch and Lomb obj. $\frac{1}{6}$, oc. 1.

FIG. 4. Same animal. Weigert fibrin stain of an injured glomerulus. The necrotic area contains a large thrombus of fibrin. Three tubules are also seen filled with hyaline casts which take the fibrin stain. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

PLATE 7.

FIG. 5. Same animal. A group of tubules, lying just beneath the capsule, their lumina filled with red blood cells. Other tubules contain hyaline casts. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.

FIG. 6. Same animal. An almost completely destroyed glomerulus. There is a marked infiltration of the glomerulus and the surrounding area with leucocytes, both polymorphonuclear and lymphocytic. Bausch and Lomb obj. $\frac{2}{3}$, oc. 1.



A B

FIG. 1.

FIG. 2.

(Wilson and Oliver: Production of specific antisera. III.)

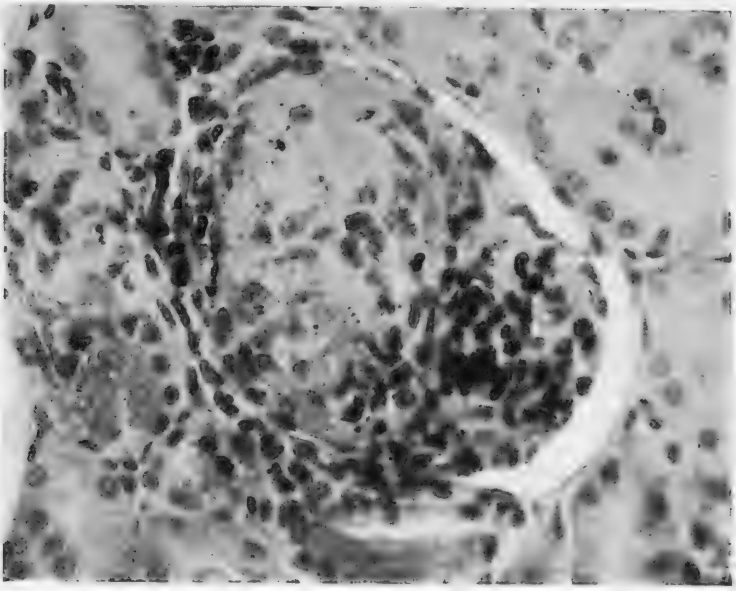


FIG. 3.

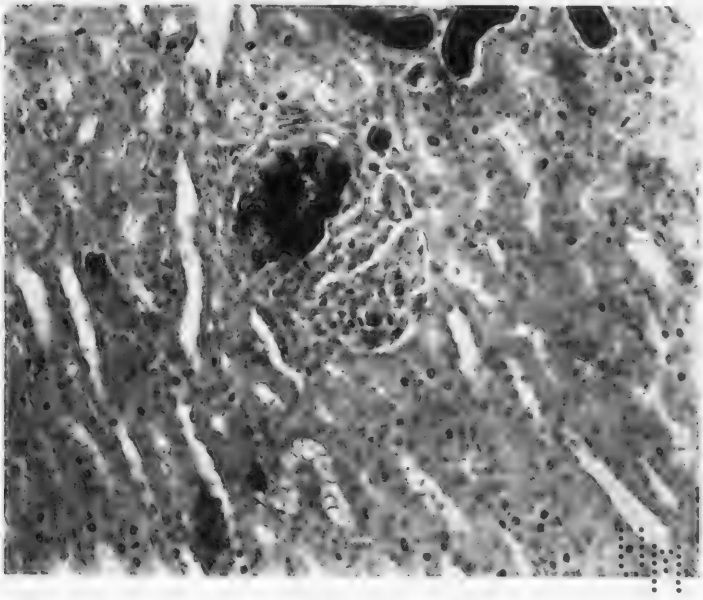


FIG. 4.

(Wilson and Oliver: Production of specific antisera. III.)

FIG. 5.

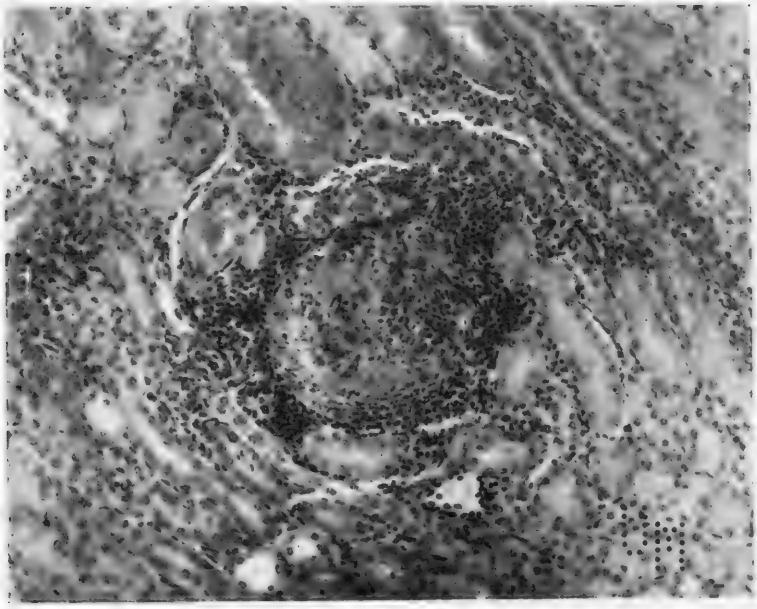


FIG. 6.

EFFECT OF INTRABRONCHIAL INSUFFLATION OF ACID.

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The resemblance between the lesions of influenzal pneumonia[1] and those produced by the inhalation of pulmonary irritating gases[2] suggested a series of experiments which might assist in the interpretation of the characteristic influenzal lesions. Many of the war gases, particularly those which act as pulmonary irritants, contain free chlorine or liberate it when they are decomposed. The weight of evidence[3] also indicates that the lesions which these gases produce depend in part upon the halogen radical, and that variations in the lesions correspond with variations in the halogen concentration as well as with the portion of the respiratory tract attacked. Moreover, it is conceded that the decomposition of these gases is associated with the formation of hydrochloric acid. The experiments now reported show that intrabronchial insufflation of hydrochloric acid solutions causes similar lesions, and these are also identical with the lesions disclosed in the examination of the respiratory organs of individuals who die of influenza.

EXPERIMENTAL.

At first isotonic saline solution with hydrochloric acid added to a strength of 1 per cent was used. By intrabronchial insufflation 5 cc. of this solution were forced into the pulmonary alveoli of rabbits previously anesthetized completely. Immediately a blood-stained,

[1] Winternitz, M. C., McNamara, F. P., and Wason, I. M., Pathology of influenza, New Haven, 1920.

[2] Winternitz, M. C., et al., Collected studies on the pathology of war gas poisoning, New Haven, 1920.

[3] Lillie, R. S., Clowes, G. H. A., and Chambers, R., *J. Pharmacol. and Exp. Therap.*, 1919, xiv, 75.

frothy fluid appeared at the mouth and nostrils, and as the catheter was removed convulsions developed. Death followed within 3 to 5 minutes.

At autopsy the very voluminous lungs filled the pleural cavities and covered the cardiac area. Occasionally the most affected lobe was collapsed, the remainder being voluminous. The glistening pink or pinkish gray pleural surfaces were tense and frequently showed hemorrhages which varied in size and shape. While all the pulmonary tissue was increased in volume, usually the most marked increase was localized on one side, even in a single lobe made conspicuous by the pleural hemorrhages. On section the lungs poured forth a large quantity of straw-colored or blood-stained fluid. After the fluid was washed away hemorrhages similar to those noted upon the surface were found within the pulmonary tissue. Frequently the peribronchial and perivascular sheaths were prominent—translucent, watery, gray, and thickened. The trachea and the larger bronchi contained blood-stained fluid, and hemorrhage into the mucosa was common. Histologically, as might be expected, there was little change. Death occurred too quickly to permit anatomical manifestation of the disturbance in the balance of cell function. There were a thick, colloid-like precipitate within the alveoli and edema of the interstitial, periarteriolar, peribronchial, and perivascular structures. There were also hemorrhages which involved both the pleural interstices, the perivascular and peribronchial tissues, and, in some instances, the alveoli. Edema followed insufflation of the hydrochloric acid solution much more quickly than it follows the inhalation of chlorine; otherwise the pathological changes were identical.

The acute cause of death after the insufflation of hydrochloric acid may not be explained by the factor of acidity alone, because the same quantity of acid may be given rabbits intravenously with impunity. Consequently, death must be ascribed to some local effect. From the fact that other acids, for example, carbolic acid, produce thrombi when directly applied to tissues, it seemed possible that coagulation of the blood within the alveolar capillaries followed the acid insufflation, and such a local coagulative action was shown to take place. The process will be described in detail in a subsequent paper.[4]

[4] Winternitz, M. C., Smith, G. H., and McNamara, F. P., *J. Exp. Med.*, 1920, xxxii, 211.

In subsequent experiments in which the concentration of acid was reduced to 0.25 per cent, sudden death was rarely observed; the majority of the animals came out of the anesthesia and later showed no untoward symptoms, unless pulmonary infection had occurred. Similar results followed the use of acids as dilute as 0.1 per cent. The gross and microscopic pictures found, even in animals of the same species, varied greatly, and it would not be useful to differentiate the effects of the two more dilute solutions mentioned. In general, the lesions caused by the weaker acids are less extensive and the likelihood of acute death is minimal.

With concentrations of acid as divergent as 1 per cent and 0.25 per cent variations in the effects are quantitative rather than qualitative. In a few instances death occurred shortly after the insufflation of the 0.25 per cent acid, possibly because of individual susceptibility. In such circumstances it is difficult to recognize any difference in the effects of a stronger and a weaker acid solution. However, when insufflated animals are killed after a period of only 15 minutes, it is found that the 1 per cent solution has excited the greater accumulation of fluid in the bronchi and trachea, and the fluid is more intensely stained with blood. In both instances the degree of edema is much the same. The hemorrhages, so prominent on the pleural surfaces and in the perivascular sheaths after the insufflation of a strong acid solution, are much less marked after the administration of a more dilute one. The picture described below is that secured with the less concentrated acid.

Animals killed at intervals after acid insufflation show a progressive localization of the changes within the lung. The less severely damaged areas lose their congested, edematous condition and assume a pale pink, delicate appearance. The more involved areas, because of their volume, their consolidation, and the dulling of their serous surfaces, contrast sharply with the less injured tissue. Within 48 hours the consolidated areas, red at first, become paler, even quite gray. They are usually wedge-shaped, most frequently involve the right lower lobe, and may extend from its lower border to the hilum. Occasionally the right lower lobe alone is involved. Frequently the process extends from the right lower to the left lower and the right middle lobes. Generally the line of demarcation between the dark red consolidated zone and the neighboring zone is not clear either with regard to consistency

or color. Upon section through such a consolidated area all structure is obliterated by the dark red color, but as the hilum is approached the architecture may become more distinct. Here alveoli distended with fluid can be seen and an occasional small hemorrhage. Later the fluid exudate becomes granular and small plugs protrude above the cut surface. At this stage the lung is less uniformly pigmented; gray patches interspersed with brownish ones and small, dark brown foci may occur.

How early this reaction takes place is difficult to determine histologically. Serum, we know, appears in the alveoli almost immediately after the insufflation, and is associated with an intense vascular congestion. In local areas also hemorrhage into the interstitial tissue and the alveoli may be prominent. The subsequent exudative changes within the lung vary with the extent and the intensity of the initial damage. Within 48 hours there appear in the lung wedge-shaped areas which show a sterile fixation of the involved tissue now devoid of nuclei. With a less marked degree of damage the alveolar walls presenting nucleated cells are distinguishable, and their lumina are filled chiefly with erythrocytes. The extreme changes will be presented in detail in a subsequent communication.[4]

The inflammatory reaction associated with milder grades of injury deserves emphasis. Within 8 hours after the damage the epithelium of the bronchioles and of the atria becomes converted into a red, ribbon-like strand devoid of nuclei. This, often thicker than the original epithelium, is raised like a blister from its basement membrane by a serofibrinous exudate in which polymorphonuclear leucocytes are found occasionally. The dilated atria and the alveolar ducts, lined with red, are conspicuous. In small areas the thickened alveolar walls show no cell nuclei. Atelectasis is not present and the degree of dilatation of the alveoli varies. The alveoli are filled with an exudate rich in serum and fibrin which contains desquamated alveolar cells. Later to this exudate are added polymorphonuclear cells which earlier were more conspicuous not only in the alveolar walls, but also in the sheaths of the bronchi and vessels. Red blood cells are also found, and, in fact, all grades of exudate occur, from those composed entirely of closely packed erythrocytes to those in which these cells are scanty or absent altogether.

The dilatation of the alveolar ducts and the hyalinosis of the bronchiolar epithelium after acid insufflation are especially noteworthy. These phenomena were characteristic both of the lesions subsequent to influenzal pneumonia and of those after exposure to corrosive gases. The terminal bronchioles are converted into conspicuous, irregular sacs, which may compress the adjacent alveoli. The marked hyalinosis, it is clear, originates from an acute necrosis of the epithelium. Nuclei can often be seen at the base of the ribbon, and in the earlier stages cell demarcation is evident. Later, when nuclei and cell boundaries disappear this membrane grows by accretion with the addition of fibrin or other material, so that ultimately it becomes thick and tends to occlude the distended bronchioles and ducts.

SUMMARY.

Acid administered to rabbits by intrabronchial insufflation causes an immediate and extreme damage of the lung tissue. Within certain limits the degree and extent of the injury vary according to the concentration of the acid. With the greater concentrations death occurs promptly, almost immediately, and the lethal process has associated with it a decreased permeability of the pulmonary vessels. The latter fact has been confirmed by postmortem arterial injections. With weaker solutions the results, grossly and histologically, resemble those noted after influenzal pneumonia and gas poisoning. Thus, there is destruction of the epithelium of the bronchioles, the alveolar ducts, and the alveoli. The extent of the damage to the alveolar walls varies. Exudation occurs into the alveolar, interstitial, perivascular, and peribronchial tissues. Primarily this exudate is serous, but a rapid deposition of fibrin occurs, and later polymorphonuclear leucocytes and erythrocytes accumulate. In different animals, or in different portions of the same lung, there may be consolidations of different types, serofibrinous, hemorrhagic, or purulent, with or without destruction of the alveolar walls. Subsequent changes relate to the organization of the exudate, necrosis, proliferation of the epithelium in the alveoli and bronchi, and, finally, to the regeneration of the pulmonary parenchyma.

Experiments now in progress indicate that similar changes can be induced by various acids, both inorganic and organic. Experiments also show that similar changes are produced in other species of animals but that species differ in their resistance according to the acid and the concentration in which it is employed.

CONCLUSIONS.

Intrabronchial insufflation of weak solutions of hydrochloric acid, from 0.1 to 0.25 per cent, in rabbits causes an inflammatory process which resembles that encountered in influenza and after the inhalation of toxic gases. There are intense edema and congestion of the lung with hemorrhage, dilatation of the alveolar ducts and bronchioles, hyaline necrosis of the epithelium of these structures, and lobular, pseudolobular, and even lobar types of consolidation, which are more frequently serofibrinous but may be hemorrhagic or even purulent.

EPITHELIAL PROLIFERATION FOLLOWING THE INTRABRONCHIAL INSUFFLATION OF ACID.

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PLATES 8 TO 11.

(Received for publication, March 31, 1920.)

Hydrochloric acid insufflation into the bronchi of rabbits causes necrosis of a degree which varies with the amount and the strength of the solution employed. With extensive damage large areas of the lung undergo fixation, and hemorrhagic infarcts may develop. A more superficial injury results in a sterile pneumonia, and the repair of the pulmonary damage begins quickly. Coincident with the formation of granulation tissue and organization of the exudate, there is a proliferation of alveolar and bronchiolar epithelium. Overproduction of the epithelium occurs with the formation of polypi in the bronchioles, and even extension of the epithelium into surrounding tissue. Although invasion of preexisting healthy tissue by the proliferating epithelium has not been observed, the ultimate results of the process have not been studied. At this time only the early changes will be presented.

Epithelial Changes after Influenzal Pneumonia.

Reference has been made elsewhere[1] to the resemblance of the lesions which follow the intrapulmonary application of acid to those associated with influenzal pneumonia, and here it may be pertinent to mention the unusual epithelial changes not infrequently seen after fatal influenzal pneumonia. Alveoli lined by large, actively dividing cells are numerous, and desquamated cells only are found in the lumina. Other portions of the lung present different stages of acute reaction,

[1] Winternitz, M. C., Smith, G. H., and McNamara, F. P., *J. Exp. Med.*, 1920, xxxii, 199.

or even of organization. The epithelium of the larger air passages —trachea, bronchi, and bronchioles—shows active regeneration. In the bronchioles especially the epithelium may not only grow over a granulating exudate in the lumen, but may invade the surrounding alveoli. It is difficult to differentiate such a cellular reaction from an invasive epithelial neoplasm. Similar reactions have been observed after the intrabronchial insufflation of hydrochloric acid.

Epithelial Changes after Intrabronchial Insufflation of Hydrochloric Acid.

The experimental procedure here was identical with that employed previously, and, therefore, need not be described in detail. Rabbits were insufflated with 5 cc. of 0.25 per cent hydrochloric acid.[2] The description of the lesions consequent to such a procedure follows.

Proliferation of the Alveolar Epithelium.—This is seen as early as 36 hours after insufflation; in 52 hours many alveoli have an entirely new layer of lining cells. As a rule, these alveoli contain but little exudate. Serum, occasional fibrin strands, or erythrocytes occur, but large, pale-staining, polyhedral cells are more frequent. The alveolar space is small, attributable in part to the size of the lining cells. These large cells with granular protoplasm and vesicular nuclei often show mitoses. Occasionally a syncytial-like mass of newly formed epithelial cells is found. The karyokinetic figures appear quite regularly. Multipolar division is not encountered. Where many alveoli within a local area have a new epithelial lining, the intense blue staining of the area attracts attention, even under low magnification.

Proliferation of the Bronchiolar Epithelium.—The bronchiolar epithelium affords the best opportunity to study the reparative process. The acid, it will be recalled, kills the epithelium, which then stains intensely with eosin as a red ribbon. For some time this material adheres to the basement membrane, and, by the addition of fibrin or other substance, it may become even more conspicuous than in earlier stages. Gradually, however, it separates from the basement membrane and, folded upon itself, lies in the lumen. If the necrosis has not extended below the basement membrane and a few of the cells have been spared, these quickly begin to divide, and at the end

[2] Before insufflation the animals were completely anesthetized.

of 36 hours a portion of the bronchiolar wall is lined by young cells. These cells are conspicuous because of their size, the homogeneity of their protoplasm, the variation in the density of the nuclei, and the frequency of mitotic figures. The dead epithelium is often separated from the basement membrane by the development of these young cells (Fig. 1).

The necrosing action of the acid may extend through the epithelial lining of the bronchioles into the surrounding tissue. When this occurs the young epithelium finds an obstacle to its regeneration along the basement membrane. Where normally there should be a single layer of cells, several are found in a sector of a tubule in which the basement membrane is intact. When these young cells reach the point where necrosis involves the deeper structures, their pathway is obstructed. Consequently, they pile up and penetrate the pulmonary parenchyma (Fig. 2). As it extends, the epithelium selects the walls of surrounding necrotic alveoli for scaffolding (Fig. 3).

The ability of this young epithelium to proliferate is extraordinary. Even when the basement membrane of a tubule is intact, it may be lined by several layers of cells within 52 hours. The dead epithelium which has been replaced is found in the lumina of the bronchioles, and there may provide a surface for the extension of the new epithelium. A picture results similar to that obtained in tissue cultures. The bronchiole, relined with an epithelial coat several times as thick as normal, shows a mass of young cells, in contact with the epithelium at one point, extending over the dead membrane within the lumen. Presumably the young epithelium feeds on the necrotic material and conditions not unlike those present in tissue cultures provide for the proliferation (Fig. 4).

This source of nutriment is merely temporary, of course, and the new epithelium would die if more favorable conditions were not provided. The invasion of fibroblasts and capillaries may supply such a deficiency (Fig. 5). In Fig. 5 the stroma has penetrated the basement membrane of the tubule to afford the necessary scaffolding and vascularization of the epithelium, and the result is a typical epithelial polyp in the bronchus. This followed within 8 days after the acid insufflation, and the epithelium of the bronchus, both on the basement membrane and in the polyp, shows continued evidence of proliferation.

Proliferation Following Damage of Large Areas of Tissue.—The most striking reparative processes are those encountered where relatively large areas of tissue have been killed by the insufflated acid. Here the architecture is still discernible. The absence of polymorphonuclear reaction is remarkable; aside from some coagulated material, the nature of which is indeterminate, the lumina of alveoli and bronchi remain free. Proliferation of the granulation tissue from the surrounding healthy lung rapidly changes these areas. The epithelium is very active and grows into the dead material along the walls of the bronchioles and alveoli. Attempts to form new alveoli are noted at the periphery of such a necrotic zone. These alveoli are usually small, lined by high epithelium, and separated from each other by fresh granulation tissue. The picture resembles that of a fetal lung.

In other places epithelial activity is less uniform. Irregular clefts and cavities, lined by rapidly dividing epithelium, are found in the newly formed stroma (Fig. 6). The picture may easily be confused with that of a malignant neoplasm.

The extent of the epithelial proliferation varies in different individuals. The results described above represent the most marked changes in a relatively large series of animals studied as late as 10 days after insufflation. In every instance the proliferation, even when extensive, was regular. Direct division of cells was not observed and the mitoses were normal without evidence of variation in the chromatin content of the daughter cells. Nuclear division without corresponding division of protoplasm has been found occasionally.

CONCLUSIONS.

The damage caused by the introduction of acid into the pulmonary parenchyma is repaired rapidly.

The depth of the necrosis determines which elements will participate in the repair. If epithelium alone is injured, epithelium alone takes part in the repair. When the damage involves the deeper tissue, organization by granulation competes with and impedes the development of the epithelium.

An overproduction of the epithelium occurs and may form bronchiolar polypi or extend into the peribronchial tissue.

EXPLANATION OF PLATES.

PLATE 8.

FIG. 1. 36 hours. The necrotic epithelium has separated from the basement membrane, and one sector of the bronchiole shows regenerating lining cells.

FIG. 2. 52 hours. Normal extension of the young epithelium is obstructed where the necrosing process involves the deeper tissues of the bronchiolar wall. Here the cells tend to invade the surrounding tissues.

PLATE 9.

FIG. 3. 5 days. Many layers of newly formed epithelium line the small bronchiole and at one point extend into the surrounding tissue. The alveoli also are lined by new epithelium. Mitotic figures are frequent.

PLATE 10.

FIG. 4. 52 hours. Where the necrotic material is in contact with the newly formed bronchiolar epithelium, it serves as a scaffolding upon which the young cells extend.

FIG. 5. 5 days. Vascular granulation tissue has invaded a mass of epithelium, as seen in Fig. 4, through the bronchiolar wall. The result is a typical polyp.

PLATE 11.

FIG. 6. 8 days. The newly formed alveoli to the left have the appearance of fetal tissue. On the right the necrotic lung is being invaded by granulation tissue and rapidly developing epithelium.

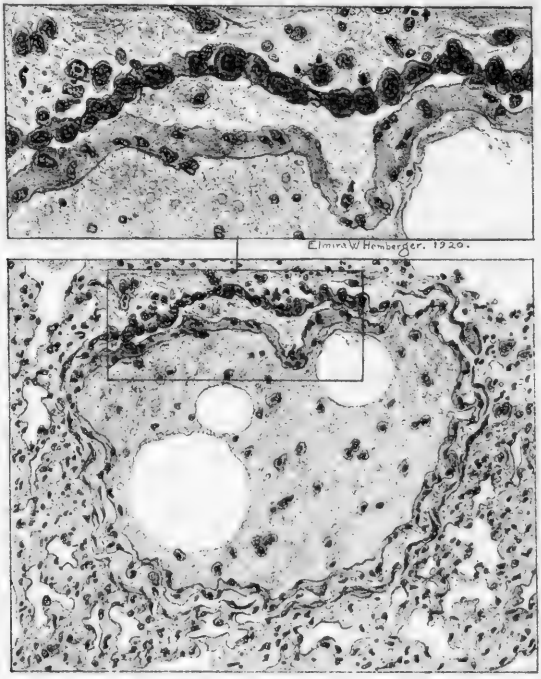

FIG. 1.

FIG. 2.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

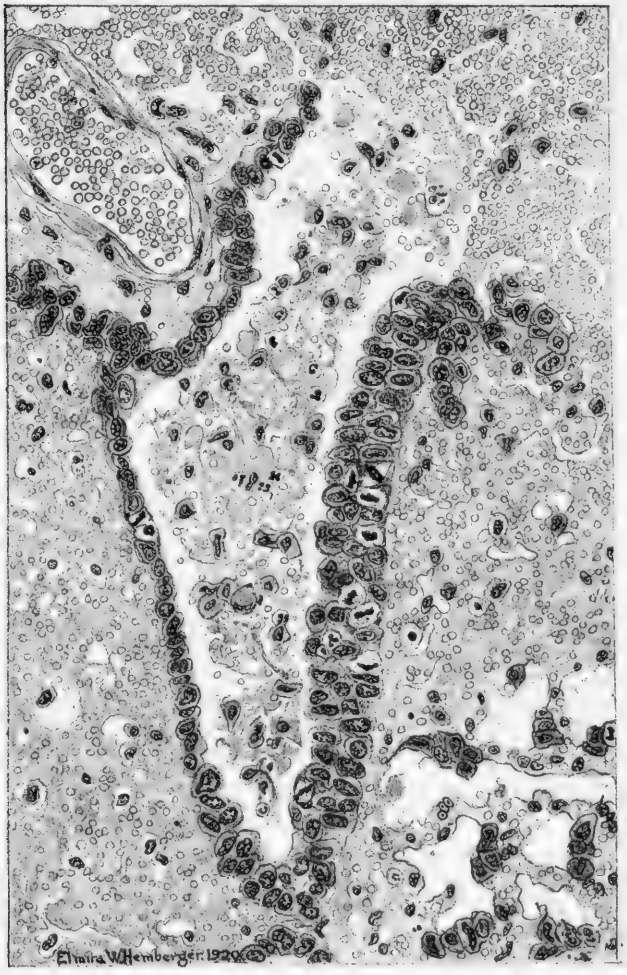

FIG. 3.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

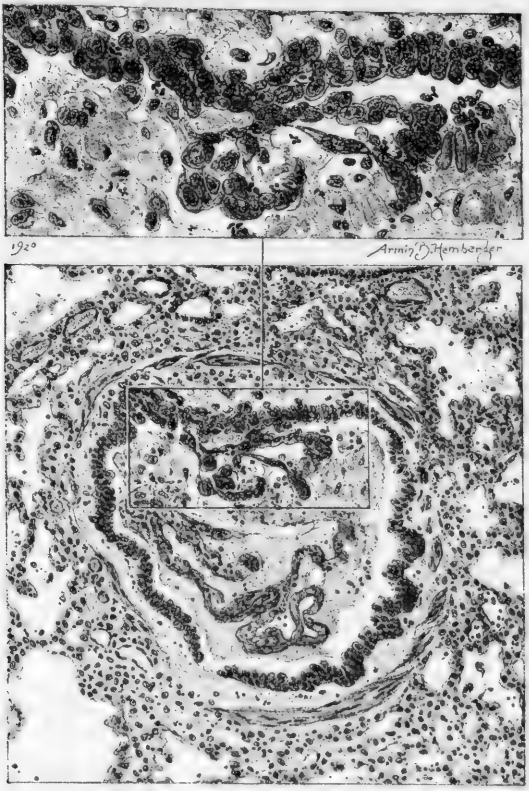

FIG. 4.

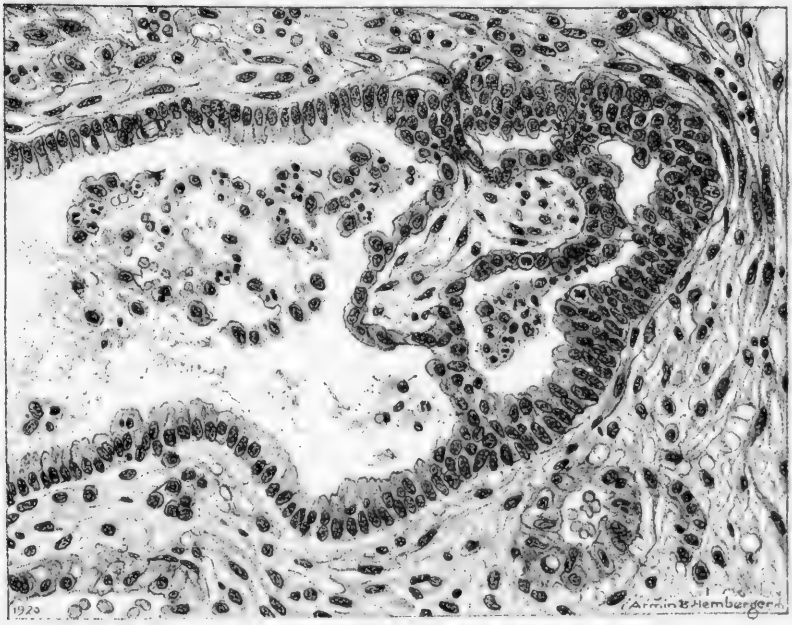

FIG. 5.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

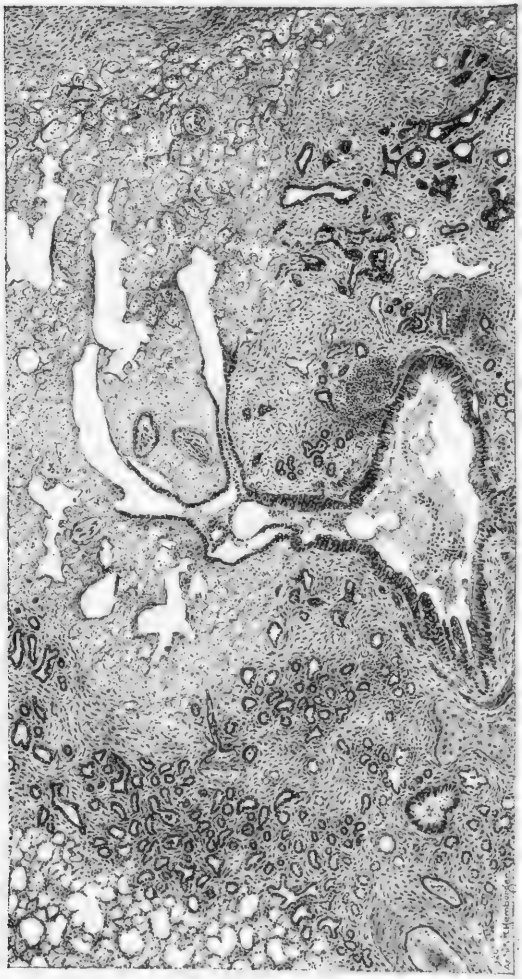

FIG. 6.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

PRODUCTION OF PULMONARY INFARCTS BY THE INSUFFLATION OF ACID.

By M. C. WINTERNITZ, M.D., G. H. SMITH, Ph.D., and F. P. McNAMARA, M.D.

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Plates 12 and 13.

(Received for publication, March 31, 1920.)

Infarcts of the lung, both in man and in experimental animals, result either from infected emboli in a normal organ or from bland emboli when cardiac decompensation or other disturbance has decreased the efficiency of the pulmonary circulation.[1] Such an explanation appeared adequate, since hitherto the infarcts so commonly found have been associated with the occlusion of a large supplying vessel with secondary extension of the thrombus into the smaller branches. A reverse of this process is shown in the experiments recorded below. The insufflation of acid into a normal lung leads to infarction, the primary process being a coagulative change in the capillary bed with secondary propagation of the clot into the larger vessels.

Extensive vascular injury in the pulmonary tissue occurs after the inhalation of irritating gases and also in influenzal pneumonia. Clinically, this is attested by the presence of blood in the sputum, and in both these conditions at autopsy hemorrhage into the lung is not infrequent. Following the inhalation of irritating gases, the pulmonary hemorrhages are often confined to the perivascular sheaths, but may involve the pleuræ, alveoli, and bronchi. These hemorrhages are rarely circumscribed in such a way as to be confounded with infarcts. In influenza the hemorrhage may be diffuse and not unlike that encountered after the inhalation of irritating gases; on the other hand, the red blood cell extravasation may be localized and definitely

[1] Karsner, H. T., and Ash, E. J., *J. Med. Research*, 1912–13, xxvii, 205.

circumscribed.[2] Indeed, the literature on the pathology of influenza refers to lesions, which, from their description, cannot be differentiated from infarcts. However, they have not been designated as infarcts, although the term "infarct-like areas" has been applied to them. Uncertainty with regard to this lesion is probably referable to the conception that bland infarcts occur only in a lung the circulation of which is impaired by cardiac decompensation or by compression of the pulmonary tissue by fluid or other substance foreign to the pleural cavity. A further difficulty in identifying these hemorrhagic consolidated areas as infarcts may have arisen through the fact that, although thrombi are frequent in the lung after pulmonary irritating gases and in influenzal pneumonia, a relation between them and infarcts has not been demonstrated. Many thrombi are unassociated with such foci, and on casual inspection at least, it is not always possible to demonstrate antemortem occlusion of the larger vessels leading to such infarct-like zones. Consequently, no explanation for the production of these infarct-like areas has been forthcoming, in spite of the fact that vascular lesions (thrombi) involving the smaller vessels in the alveolar walls and even the larger arterioles have been described by many observers.

In a previous communication[3] the results of intrapulmonary insufflation with an inorganic acid (hydrochloric) have been reported. Here it may be reiterated that the extent of the damage to the lung varies with the strength of the acid solution employed. When a 1 per cent solution of hydrochloric acid is insufflated into the lung of a rabbit,[4] the animal succumbs acutely, and during the few minutes it remains alive after the treatment, blood-stained fluid froths from the mouth. With weaker solutions the animals do not die acutely, and the destruction of the lung with subsequent inflammatory exudation is more outspoken.

[2] Winternitz, M. C., McNamara, F. P., and Wason, I. M., Pathology of influenza, New Haven, 1920.

[3] Winternitz, M. C., Smith, G. H., and McNamara, F. P., *J. Exp. Med.*, 1920, xxxii, 199.

[4] Before insufflation the animals were completely anesthetized.

Lesions in Animals That Survived Only 15 Minutes.

A few of the animals insufflated with 5 cc. of 0.25 per cent hydrochloric acid died within 15 minutes. In all of them the gross picture is identical and is illustrated in Fig. 1. The voluminous lungs are slightly congested throughout, but the most constant and marked changes occur in the lower lobes, usually the right. The increase in size is much more marked in the affected lobe than elsewhere. The lower third, and sometimes even half of the lobe, is garnet, and the color is accentuated by the glistening, smooth pleural surface through which it is seen. The discolored area of the lung is non-air-containing. On section, as on the surface, the line of demarcation is sharp and defines a wedge-shaped zone whose apex approaches the hilum (Fig. 2). Histologically, sections through such a consolidated area show a mass of well preserved erythrocytes filling the alveoli and even the bronchi. The alveolar and bronchiolar walls may still be made out; though shrunken, their cellular elements are intact and the nuclei stain (Fig. 3).

Lesions in Animals Killed at Varying Intervals after Insufflation of Acid.

Animals which survived the insufflation of acid in this concentration were killed at intervals up to 2 weeks after the operation, and examination of the lungs of such a series indicates clearly that there is a variation in the effects of the acid in animals of the same species. At times there are no areas of consolidation and discoloration like those described for the previous group, and the extent of such change varies from small foci to areas which involve the greater portion of a lower lobe. Especially in their lower half, these larger infarct-like areas often present a dull, even pale brown, surface. Above this zone, either sharply or gradually, the color becomes almost black. The surface markings are an index to the color change within the consolidated portion of the lung, and microscopic examination of the paler areas offers the most suggestive explanation of the etiology of the change. Sections from the dark, almost black portion of the consolidated lung are similar to those just described (Fig. 3). The alveoli are packed with disintegrating red blood cells, while the alveolar and bronchiolar walls are still well defined, and the cell nuclei retain

their affinity for hematoxylin. Evidence of organization and epithelial regeneration appears about the area of hemorrhagic extravasation, but discussion of this phenomenon will be deferred. The hemorrhage in the alveoli becomes less marked in sections from the paler areas. Here, the alveoli and bronchi contain a homogeneous, eosin-staining material; few red blood cells are included in the exudate. The most interesting changes occur in the alveolar and bronchiolar walls, which are swollen and often measure several times their normal width (Fig. 4). The nuclei do not stain with hematoxylin, and in the portions most affected no structure can be made out in the walls.

Striking infarct-like lesions have been produced in dogs also, by the insufflation of acid (20 cc. of 1 per cent hydrochloric acid). In this species the large size of the pulmonary vessels makes it easier to study those leading to the area of consolidation, and multiple sections frequently show them distended and occluded by a firm, red clot (Fig. 2). Histologically, in all animals, including rabbits and dogs, thrombosed vessels are found. The clot has the appearance of a propagated thrombus. It is devoid of the typical thrombotic architecture, and consists largely of red blood cells with leucocytes scattered more or less regularly among them. Occasional foci of platelets with radiating fibrin strands are present.

Explanation of Infarct-Like Areas.

The explanation for the unusual production of infarct-like areas after the insufflation of acid is not evident from the histological study of the more typical lesions. However, as has been said above, the microscopic picture of the paler brown peripheral zone that occurs when rabbits survive for several days, offers a clue toward the interpretation of the process. Here the swollen alveolar walls are necrotic (Fig. 4). The alveoli may contain serum and fibrin, but no cells—an unusual picture in the lung. The process resembles gangrene more than any other familiar lesion of this organ, and bears a strong resemblance to an anemic infarct in the spleen or kidney. The walls of the alveoli stain homogeneously with eosin; the architecture is obscured, but now and then either the lining cells or the vessel within the wall can be distinguished. Similar changes in the alveolar walls

have been described in influenza, and they also develop rapidly after the inhalation of irritating gases. It is impossible to distinguish the vessels, and frequently the blood and other constituents of the wall are so intimately fused that the individual elements can no longer be differentiated. The picture is not unlike that resulting from the application of carbolic acid to the gastric mucosa. In all probability the insufflated acid causes a fixation of the tissues with simultaneous inhibition of all cell activities. Consequently, the intermediary regressive processes dependent upon the loss of balance of the cellular ferments are lacking.

This picture results, as has been said, when the more concentrated acid is employed, and, therefore, may be considered to represent a maximal effect. Milder grades of damage also may result in thrombosis of the small vessels. If vessels in sufficient number are occluded, the resistance to the flow of fluid in the lung should be demonstrable by pulmonary arterial injection, and to test this point a series of experiments was devised.

It was found that a longer period of time than in normal subjects was required for a Berlin blue gelatin injection mass to appear on the venous side, even in animals that had succumbed within a few minutes after the insufflation of a 1 per cent solution of hydrochloric acid. The lung cannot be injected uniformly; and microscopically, in thick frozen sections, it is readily seen that many vessels are not permeated by the mass. When local, consolidated, red areas have developed, increased pressure, even after the dye has appeared on the venous side, does not result in injection of the consolidated zone. This zone contrasts sharply with the neighboring area, deeply stained by the dye in its vessels.

The injection method thus provides the means of determining the effects of the acid more minutely. The lung may appear uniformly blue, while, in fact, numerous, small areas, scattered through one or more lobes, contain none of the gelatin. On the other hand, a large area may be completely uninjected and sharply demarcated. Between these extremes there are many variations. Histological examination indicates that the acid coagulates the blood in the swollen vessels, and the phenomenon is actually demonstrated upon injection. By this method variations in the gross picture are explained through localization of the coagulative process.

Our immediate interest lies in the instances in which the action of the acid is confined to a local area. Where this occurs it is possible to show that large vessels leading to the sharply demarcated zone are distended with clot. However, these clots are not found in the animals that die within a few minutes; they are most marked after a period of several days.

From the above experiments we conclude that when concentrated acid reaches the alveolar walls, necrosis of the entire structure and thrombosis of the vessels follow. The resulting lesion depends on the extent of the necrosis and thrombosis. When a large number of vessels becomes occluded in a given area, the possibility of the propagation of the clot into the larger vessels is increased, and when this occurs, the lesion cannot be differentiated from an infarct.

Infarction in tissues with an extensive collateral circulation does not occur except under extraordinary circumstances. Therefore, one would hardly expect this process in the liver, and no really authoritative cases of infarction of the liver have been reported. Nevertheless, a liver lesion which can easily be confused with infarction has been observed recently in two cases in which the capsule of the organ was torn and the rent extended several centimeters into the parenchyma. Both individuals lived several days after they were injured. At autopsy, in each case, the hepatic tissue surrounding the clot at the point of rupture was the seat of a coagulative necrosis indistinguishable from the type of necrosis which occurs in infarcts of the spleen and kidney. When the liver was torn the smaller capillaries within the lobules were broken, and with the cessation of hemorrhage, clots formed at the mouths of these capillaries. These clots were propagated into the radicles of both the hepatic artery and portal vein, so that the extensive collateral circulation within the lobule, the safeguard against all but extraordinary conditions, was rendered ineffective. It would seem that this is a type of infarction that can result from the plugging of peripheral vessels with subsequent propagated clots into the larger supplying vascular bed. Unquestionably, this is the process by which infarcts are formed after insufflation with acid; and, in all probability, the infarct-like areas associated with influenza also result from similar damage to the intraalveolar capillaries or arterioles which undergo thrombosis.

CONCLUSIONS.

Intrabronchial insufflation of acid causes immediate necrosis of the walls of many alveoli. Thrombosis of the alveolar vessels is an associated phenomenon. When a large number of vessels becomes affected, a clot propagates rapidly into the larger supplying vessels. The resulting lesion is indistinguishable from a hemorrhagic infarct.

The infarct-like areas so frequently encountered in influenzal pneumonia, it is not unlikely, have their origin in a similar process.

Infarction depends not only upon thrombosis or embolism of the large vessels, but may be initiated by extensive damage to the capillary bed. By this process infarcts may form in organs which are normally protected by collateral circulation.

EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Infarct of the lung produced by the insufflation of 5 cc. of 0.25 per cent hydrochloric acid.

FIG. 2. Cross-section of an infarct of the lung. The larger supplying artery is occluded by a propagated clot.

PLATE 13.

FIG. 3. Histology of an infarcted area. The alveolar walls are thin and the alveoli are distended with erythrocytes.

FIG. 4. Section showing the maximal effect of acid on the lung tissue. The alveolar walls are necrotic and their vessels contain hyaline thrombi.

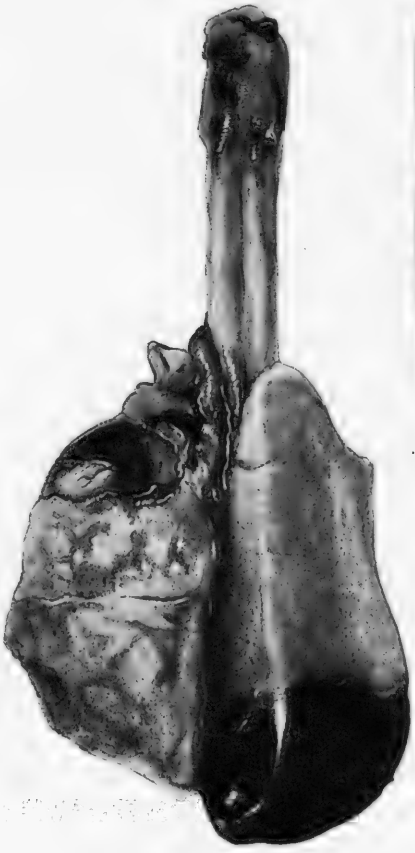

FIG. 1.

FIG. 2.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

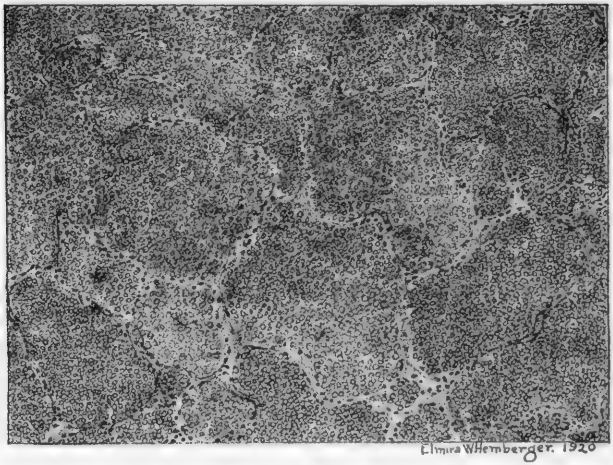

FIG. 3.

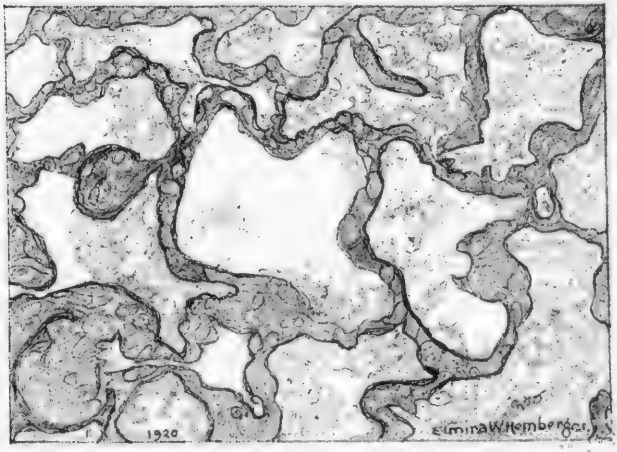

FIG. 4.

(Winternitz, Smith, and McNamara: Intrabronchial insufflation of acid.)

A STUDY OF BACILLUS PYOGENES.

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Plates 14 to 16.

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INTRODUCTION.

Bacillus pyogenes is associated with various disease processes of swine and cattle and is not infrequently found in milk. Failure to recognize it as the *B. pyogenes* described in French, Dutch, and German literature may be due to certain difficulties in its cultivation, to its close resemblance to streptococci under certain conditions, and to the fact that it is usually found in mixed culture with organisms which may mask its presence.

In France, according to Lucet (1893), next to the streptococci *B. liquefaciens pyogenes* is one of the most frequently found organisms in suppurations of cattle. In Germany Künnemann (1903) found a similar organism, which he calls *B. pyogenes bovis*, in 90 per cent of suppurations of cattle. Grips (1898) found *B. pyogenes suis* commonly present in pleuritis and peritonitis of swine. Tuff (1906) found *B. pyogenes* in over 13 per cent of milk samples examined. Glage (1902–03) found the organism third in importance to streptococci and *B. tuberculosis* as a cause of mastitis in cows. Eggink is quoted by Ward (1917, *a*) as having found *B. pyogenes* of first importance in metritis of cattle. There are no statistics as to the prevalence or distribution of the organism in America. Ward (1917, *a*) found it frequently in swine and cattle.

Lucet (1893) studied 52 cases of suppuration, cold abscesses, traumatic abscesses, and cases of septicemia, all in cows. He says: It seems that there exist in the cow special pyogenic microbes, not yet described, which are a streptococcus, a staphylococcus, and three bacilli. He names these organisms *Streptococcus pyogenes bovis*, *Staphylococcus pyogenes bovis*, *B. pyogenes bovis*, *B. liquefaciens pyogenes bovis*, and *B. crassus pyogenes bovis*. His description of *B. liquefaciens pyogenes bovis* agrees with that of the *B. pyogenes* of Grips, Glage, Künnemann, and others. Lucet gives a photograph of the organism and describes it as non-motile, liquefying gelatin slowly, not growing on potato, growing as a sediment in veal bouillon without producing turbidity, non-virulent for guinea pigs, injected into rabbits intravenously producing subaponeurotic abscesses principally in the limbs where they sometimes acquire great size but do not discharge. His *B. pyogenes bovis*

resembled the above morphologically but did not liquefy gelatin and its pathogenicity for guinea pigs was variable. *B. crassus pyogenes bovis* was a larger motile bacillus. Grips (1898) described *B. pyogenes suis* and the lesions of the pleura and peritoneum from which it was isolated. Poels (1899) described a similar organism from polyarthritis of calves and called it the polyarthritis bacillus. Künnemann (1903) described *B. pyogenes bovis* from suppurations of cattle. Glage (1902–03) made a careful comparison of *B. pyogenes suis* (Grips) and *B. pyogenes bovis* (Künnemann) and concluded that they were identical. He proposed that the organism be called *B. pyogenes*. His contention that the organisms from swine and cattle are identical has not been seriously questioned. Careful bacteriological studies have been made by Koske (1906), Berger (1908), and Holth (1908). Ward (1917, *a* and *b*) has given valuable brief summaries in English, and Glage (1913) in German.

Source of Strains.

The strains of *Bacillus pyogenes* of bovine origin employed in this study were received from Dr. Theobald Smith who supplies the following data concerning their source.

Strain I. From pneumonic lungs of a calf. Killed when 33 days old.[1]

Strain II. From pneumonic lungs of a calf. Killed when about 5 weeks old.[1]

Strain III. From pneumonic lungs of a calf. Killed when 38 days old.[1]

Strain IV. From pus filling both horns and body of uterus of a cow. Uterus obtained after slaughter of cow.

Strain V. From a similar case as that of Strain IV. Uterus contained a foul smelling fluid.

Strain VI. From purulent contents of uterus in case of prolapse of vagina and external os. Uterus obtained when cow was slaughtered.

Strain VII. From pneumonic lungs of a calf. Killed when 31 days old.

Strain VIII. From the uterine contents and ovaries of a case of purulent metritis and of central necrosis and pus formation in both ovaries. Associated with *B. actinoides*.

Strain IX. From chocolate-colored, offensive fluid contained in uterus of a cow slaughtered. Other bacteria present.

Strain X. From kidney, liver, and lungs of the fetus of Cow 259. Several other species of bacteria present in small numbers.[2]

Strain XI. From the fourth stomach, liver, and lungs of the fetus of Cow 291.[2]

Strain XII. From the liver of the fetus of Cow 339. Pure culture of *Vibrio fetus* isolated from the lungs.

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[1] It is highly probable that in these cases *Bacillus pyogenes* was secondary to *Bacillus actinoides* (Smith, T., *J. Exp. Med.*, 1918, xxviii, 333).

[2] Smith, T., *J. Exp. Med.*, 1919, xxx, 325.

Strain S 1 was isolated by Dr. Carl TenBroeck from the pneumonic lung of a case of hog-cholera. Other organisms were also present in the lung.

Cultural Study.

The known morphological and cultural characteristics of *B. pyogenes* as described by Lucet (1893), Grips (1898), Poels (1899), Künnemann (1903), Glage (1902–03), Koske (1906), Berger (1908), and Holth (1908) have been summarized by Glage (1913), Buchanan and Murray (1916), and Ward (1917, *b*). They are briefly as follows:

The organism is a small slender rod 0.2 to 3.0 microns in length by 0.2 to 0.3 microns in thickness. It is quite pleomorphic being often coccoid, club-shaped, or slightly curved. It is non-motile and produces no spores. Some authors (Glage, Künnemann) have regarded it as Gram-negative and others as Gram-positive (Berger, Holth, Olt, Ward). Berger found it Gram-positive if subjected to sufficient exposure to the iodine solution. Capsules are not produced.

It is stated by most authors that the organism grows very poorly or not at all in standard bouillon or on standard agar, and that it requires hemoglobin, blood, or serum in the medium. Good growth occurs on blood or serum agar. Coagulated blood serum is slowly liquefied beginning in about 48 hours as small depressions underlying each colony. Because of this characteristic this medium has been a favorite one for isolating the organism. Milk is coagulated in about 48 hours and the curd is subsequently slowly dissolved or digested. In liquid serum or serum bouillon growth occurs in the form of a sediment. Growth does not occur at temperatures below 24°C. but in a specially prepared nutrient gelatin of high melting point Poels found liquefaction produced by the organism growing at 26°C. Growth occurs under aerobic and anaerobic conditions. Gas is not produced in carbohydrate media. According to Pütz (1904) acid is produced. Koske (1906) reports acid production in serum litmus whey. Berger (1908) obtained no growth in serum litmus whey and does not mention acid production in lactose or dextrose bouillon but notes that milk is coagulated and soured. Holth (1908) reports acid production from dextrose, fructose, galactose, maltose, lactose, and saccharose in a special meat extract (Cibil's) bouillon but obtained no acid or visible growth in the same medium containing xylose, rhamnose, arabinose, sorbose, mannitol, sorbitol, dulcitol, or glycerol. Indole, hydrogen sulfide, and nitrites are not produced. Methylene blue, litmus, and neutral red are not reduced. The bacillus is soon killed at 57°C. and is very sensitive to antiseptics.

Our experience with the organism agrees with the above as regards morphology, oxygen requirements, growth on coagulated serum, in milk, and in serum bouillon. In plain standard veal infusion bouillon made with Fairchild's peptone, however, we have obtained fairly

good growth, at least with strains which have been in cultivation a very short time. For a while the bouillon was not clouded by the culture but after cultivation for several months bouillon was distinctly clouded in 24 hours by most strains.

The production of hemolysis in blood agar by *Bacillus pyogenes* appears not to have been noted heretofore. In standard veal infusion agar plus 5 to 10 per cent of defibrinated horse blood there appear after incubation for 20 to 24 hours very small zones of hemolysis about very minute deep colonies. The colonies are often visible only under the low power of the microscope. In 48 hours the deep colonies are still quite small biconvex discs about 0.3 mm. in greater diameter but are easily seen macroscopically. The hemolyzed zones are clear, well defined, colorless, and of the beta type (Smith and Brown, 1914–15; Brown, 1919), about 1.5 to 2 mm. in diameter (Fig. 1). Isolated surface colonies do not appear so readily on the plate under aerobic conditions and the zone of hemolysis may be hardly visible. If the plate is sealed, however, individual surface colonies grow more readily and produce zones of hemolysis similar to those of deep colonies. If the blood agar plate is streaked so that many small surface colonies appear in the line of the streak, hemolysis appears beneath the streak. The individual surface colonies are very small convex colorless droplets much like those of *Bacillus influenzæ*.

We have sought to determine whether *Bacillus pyogenes* is hemoglobinophilic or whether it may be dependent upon other substances in blood for growth.

Freshly drawn horse blood was allowed to clot and a clear straw-colored serum was obtained as nearly free from hemoglobin as possible. This was used in serum agar plates.

Another portion of the same blood was defibrinated, and the corpuscles were washed repeatedly with sterile physiological salt solution. Some of the washed corpuscles were used in washed corpuscle agar plates.

Some of the washed corpuscles were laked with sterile distilled water and the corpuscle stroma removed by centrifugation. Care was taken to centrifuge the laked blood corpuscles until the supernatant hemoglobin solution no longer gave a clouding reaction with salt (Brown, 1919[3]). The hemoglobin solution so obtained was used in hemoglobin agar plates.

[3] Brown (1919), p. 67.

The corpuscle stroma obtained by centrifugation of the laked corpuscles was washed repeatedly in sterile distilled water and in salt solution until no visible trace of hemoglobin remained. The stroma suspension was used in stroma agar plates.

Each strain of *Bacillus pyogenes* was inoculated into the depths and streaked onto the surface of plates of the following media: (1) blood agar; (2) serum agar; (3) washed corpuscle agar; (4) hemoglobin agar; (5) stroma agar; (6) plain agar. Every precaution was taken to insure uniformity of conditions, such as use of the same lot of agar throughout, inoculation of one plate after another in the same manner and with the same amount of material. The plates were inoculated from fresh plain bouillon cultures. Observations were made as to the amount of growth, size and number of colonies, and morphology of the organisms within the colonies. In every case the best growth was obtained in blood agar. Next best was the growth in washed corpuscle agar and in serum agar. In the majority of cases better growth was obtained in stroma agar than in hemoglobin agar. Little or no growth occurred in plain agar. The results indicate that serum is of as much importance as corpuscles, and that hemoglobin is probably the least essential of the blood constituents for the growth of this organism. That hemoglobin does not satisfy the requirements of *Bacillus pyogenes* as it does those of *Bacillus influenzæ* is indicated by the following experiment. A blood agar plate was inoculated in the depths and also streaked with *Bacillus pyogenes*. At a point near the streak a large zone of laking was produced by depositing a bit of saponin on the surface of the medium. Both deep and surface colonies within the zone were no larger and no more numerous than elsewhere in the plate (Fig. 1). Under similar conditions colonies of *Bacillus influenzæ* grew more luxuriantly in and near the zone of laking than elsewhere in the plate. A similar result was obtained by producing zones of laking by directing a stream of carbon dioxide against the bottom of the plate until a spot was frozen. The blood used for such experiments must be fresh; otherwise there will be sufficient free hemoglobin in the serum to obliterate the difference in growth between that in the laked zone and elsewhere.

The fact that colonies of *Bacillus influenzæ* grow more luxuriantly in the vicinity of colonies of hemolytic streptococci and staphylococci has

been known for many years (Grassberger, 1897). According to Davis (1917) there is involved in this phenomenon not only hemoglobin but also a vitamine which is supplied by the foreign organism or may be supplied by fresh sterile vegetable or animal tissues. We have not found the growth of *Bacillus pyogenes* colonies to be augmented by proximity to colonies of other organisms.

That blood is not absolutely necessary for the growth of *Bacillus pyogenes* is shown by the fact that it grows very well on Dorset's egg medium. On this medium the colonies, especially those on the upper half of the slant, produce little pits like those produced on coagulated serum. The organism also grows fairly well on a medium consisting of three parts of white of egg plus one part of standard veal infusion bouillon, slanted and coagulated in the inspissator. On this medium the streak of growth also produces some depression as on coagulated serum. Even an old laboratory strain of *Bacillus influenzæ* which has become quite easy to cultivate grows very slightly on Dorset's medium and not at all on the egg white medium.

Staining and Morphology.

Bacillus pyogenes stains well with dilute carbolfuchsin or with Löffler's methylene blue but we have obtained the best results with the Gram stain. Under the influence of prolonged decolorization with alcohol the organism may not retain the violet stain so tenaciously as do staphylococci, but when stained on the same slide with *Bacillus influenzæ*, *Bacillus coli*, the meningococcus, or other recognized Gram-negative organisms there can be no doubt as to *Bacillus pyogenes* being clearly Gram-positive. The disagreement in the literature as to the Gram staining of the organism may be due to the many methods in use for applying and making up this stain and the poor keeping qualities of some of them. It is also pointed out by Olt (1908) that the dead organisms found in old exudates are Gram-negative whereas the living organisms are Gram-positive. We have used Stirling's aniline gentian violet, but 1 or 2 per cent of the dye by weight rather than 5 per cent has been found sufficient. Exposure of films to the violet stain for 10 seconds, Lugol's solution for 15 to 30 seconds, decolorization in alcohol until no more color appears to be given off,

and counterstaining for 10 seconds in aqueous safranine has always given good results.

The various media employed have afforded opportunity to observe the pleomorphism of *Bacillus pyogenes*. Four or five more or less distinct forms and their intermediate stages are recognizable.

1. Bacillary Form.—These are small, short, homogeneously stained Gram-positive bacilli occurring singly (Fig. 2).

2. Fusiform Form.—In this form there are one or two strongly Gram-positive central granules while the ends of the bacilli fade out and take the counterstain to some extent. These are often slightly curved, and superficially resemble *Bacillus acne* though smaller (Figs. 3 and 4).

3. Diphtheroid Form.—These are bacilli of irregular length and contour, some clubbed, containing deeply stained bands or granules irregularly placed (Fig. 6).

4. Streptococcoid Form.—These appear exactly as Gram-positive diplococci and streptococci in small clumps and short crooked chains. If, as may be supposed, these cocci are but the granules of the diphtheroid form which have assumed a very regular size, form, and arrangement, the matrix forming the remainder of the bacillus is invisible to the eye though the photographs reveal a faint matrix which probably retained a trace of the red counterstain (Figs. 7 and 8).

5. Filamentous and Branching Form.—The bacilli appear drawn out with irregular diameter. There are few definite deeply staining granules but in some filaments there may be a deep violet portion blending gradually with a faintly stained or Gram-negative portion. Definite buds and branches were occasionally found, especially in Strain IV (Figs. 5 and 6).

Cultures often show a mixture of the above forms. Some strains have more of a tendency to assume one form and others another, but all the strains studied have produced all the various forms at one time or another. The streptococcoid form may be so definite and so habitual with certain strains that the organism may be mistaken for a streptococcus. We have spent some weeks studying what was thought to be a very unusual minute hemolytic streptococcus only to discover that we were working with *Bacillus pyogenes*.

In observing the morphology of the organism on various media we hoped to discover the factors which determined the variations in form, but the hope was only partially realized. In the lesions of animals the organism appears in the bacillary form with a tendency to be more granular in older lesions. In bouillon and serum bouillon the bacillary form predominates though any or all of the other forms may also be present; the streptococcoid form is least likely to appear. The latter form was encountered most commonly on washed corpuscle agar, stroma agar, and especially on hemoglobin agar. On blood agar there was a mixture of bacillary, granular, and streptococcoid forms. The growth on serum agar was bacillary. Long filamentous and branching forms were obtained in serum bouillon and in the condensation fluid of coagulated blood serum. Fusiform bacilli appeared most commonly in milk and in bouillon containing fermentable sugar. Our study has produced the impression that the filamentous and streptococcoid forms represent the two extremes of pleomorphism. The strains which produced filamentous forms most readily, *e.g.* Strain IV, produced streptococcoid forms with difficulty, while other strains, *e.g.* Strain X, produced streptococcoid forms readily but filamentous forms rarely. The forms most commonly encountered are the bacillary and fusiform.

Fermentation.

In fermented bouillon plus 10 per cent of sterile horse serum and 1 per cent of the test substance all the strains of *Bacillus pyogenes* produced acid from dextrose, saccharose, lactose, and xylose but not from raffinose, inulin, mannitol, and salicin. Holth (1908) reports that galactose, fructose, and maltose are also fermented. We differ from him in regard to xylose. Our fermented bouillon containing the test substances was probably more favorable for the growth of the organism than was his meat extract bouillon.

Fermentation tubes were employed for the tests and the contents of both the open bulb and the closed arm were titrated for acid after incubation of the cultures for 7 days. The results recorded in Table I show that the acidity of the bulb was much higher than that of the closed arm, little or no acid often being produced in the latter. *Bacillus pyogenes* is completely agglutinated by normal horse serum in

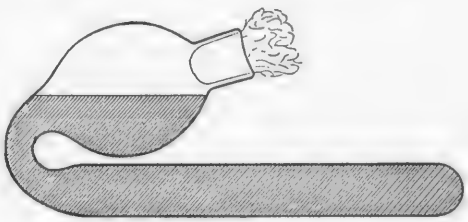
dilution of 1:100. In dextrose serum bouillon the arm of the fermentation tube remains clear and most of the growth is in the form of a sediment in the neck of the tube. If, however, the fermentation tube be incubated in the horizontal position (Text-fig. 1) an abundance of

TABLE I.

Fermentation Reactions.

Strain No.	Xylose.		Dextrose.		Lactose.		Saccharose.		Raffinose.		Inulin.		Mannitol.		Salicin.	
	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.	Bulb.	Arm.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I	3.85	2.15	3.35	1.4	4.25	2.45	1.95	0.8	0.9	0.4	1.1	0.75	1.5	0.55	1.1	0.9
II	4.1	1.9	3.55	2.1	5.35	2.1	3.25	1.9	0.75	0.35	0.9	0.5	0.65	0.75	0.7	0.7
III	3.8	2.05	3.35	1.8	5.5	3.05	2.3	1.45	1.0	0.65	0.6	0.6	1.0	0.65	0.7	0.6
IV	4.0	1.9	3.9	1.2	3.35	0.85	2.75	0.9	0.85	0.4	0.75	0.55	1.0	0.65	0.75	0.4
V	3.55	2.2	3.7	1.95	3.9	1.65	2.8	1.6	1.15	0.6	0.9	0.4	0.75	0.5	0.7	0.4
VI	4.8	2.55	3.4	2.1	2.0	1.6	2.65	1.05	0.95	0.55	1.05	0.7	0.8	0.4	0.65	0.65
VII	3.35	2.55	2.6	2.2	4.05	2.35	3.75	1.65	1.2	1.0	1.3	1.05	1.15	0.08	1.6	0.85
VIII	4.4	3.2	4.0	1.5	4.6	2.5	3.2	2.7	0.65	0.45	1.0	0.9	0.95	0.5	1.1	0.65
IX	2.9	1.2	2.65	1.45	1.85	1.1	2.85	1.85	0.5	0.4	0.8	0.4	0.4	0.5	0.75	0.45
X	3.1	0.7	3.4	0.85	3.9	1.4	3.25	1.35	0.85	0.5	0.85	0.35	0.6	1.2	0.65	0.5
XI	3.75	1.8	3.85	1.5	2.8	1.05	3.4	1.15	1.1	0.8	1.2	0.4	0.75	0.45	0.8	0.6
XII	3.35	2.35	3.0	1.35	1.8	1.2	3.5	1.3	1.0	0.65	0.9	0.55	0.75	0.7	0.9	0.65
S 1	3.75	1.35	4.15	1.4	4.1	1.25	1.8	0.6	1.45	1.0	1.2	0.6	0.6	0.4	1.0	0.9

The figures indicate per cent normal total titratable acid.



TEXT-FIG. 1. Fermentation tube placed in the horizontal position.

acid is formed in the arm as well as in the bulb. These facts suggested for the moment that failure to ferment in the arm might be due to the mechanical effect of agglutination. It was discovered, however, that in plain dextrose bouillon without serum the arm was well clouded by

growth but little or no acid was produced in it. The effect of difference in oxygen tension was next considered. The closed arm of a fermentation tube of sterile bouillon containing 1 per cent of a 1:1,000 aqueous solution of methylene blue reduced by autoclaving remains colorless for weeks if incubated in a vertical position. The arm of a similar tube incubated in the horizontal position is well colored in a few hours. In the following experiment tubes of dextrose bouillon covered by 5 cc. of vaseline were placed in a boiling water bath for 30 minutes at the end of which time the methylene blue in the control tube was colorless. The other sealed tube was cooled and inoculated by means of a capillary pipette through the layer of vaseline. There was good growth in both inoculated tubes.

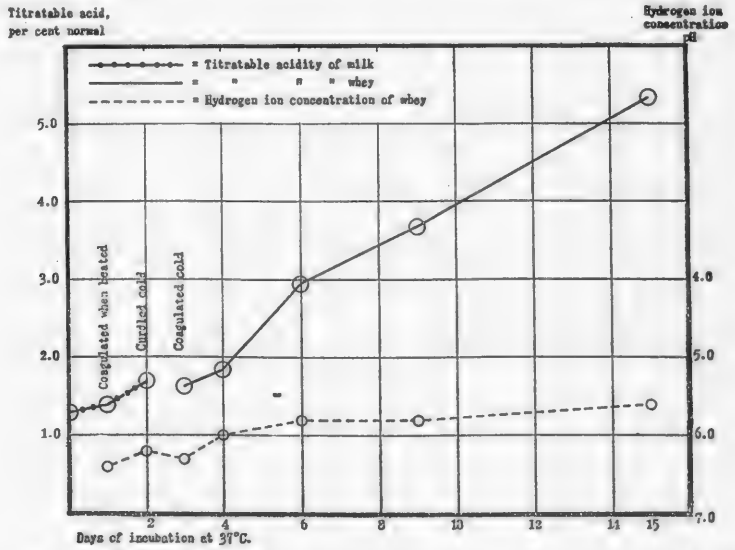
Strain No.	Sugar.	Relation to air.	Incubated 7 days.	
			Titration.	pH
			per cent	
X	Dextrose.	Not sealed.	3.9	5.1
	"	Sealed.	2.1	6.2
Sterile methylene blue.	"	"	Remained colorless.	

The titratable acidity is expressed as per cent normal acid.

It is to be noted that the behavior of *Bacillus pyogenes* as regards fermentation of sugars in relation to oxygen tension appears at variance with that of other organisms, notably those of the colon group. The idea, possibly correct in the case of most organisms, is prevalent that facultative anaerobic organisms are able to satisfy their oxygen requirements by breaking down fermentable sugars and that in such cases fermentation is likely to be more vigorous or at least more apparent under anaerobic conditions than in the presence of free oxygen. Some strains of *Bacillus coli* may produce an alkaline reaction in the bulb and acid and gas in the closed arm of the fermentation tube containing saccharose bouillon. *Bacillus cloacæ* may react similarly in lactose bouillon. The alkalinity or lower acidity of the bulb, however, may not be due to diminished fermentative activity but to the simultaneous production of large amounts of alkali.

Growth in Milk.

One of the cardinal cultural characteristics of *Bacillus pyogenes* is its ability to coagulate milk and slowly digest the curd. If an indicator solution such as rosalic acid and china blue (Bronfenbrenner, 1918–19) or bromocresol purple (Clark and Lubs, 1917, *b*) is placed in the milk, the latter is seen to become acid and remain acid for at least 3 weeks during which time much of the coagulum disappears. The question arises as to whether the coagulation of milk is due to acid or to an enzyme.



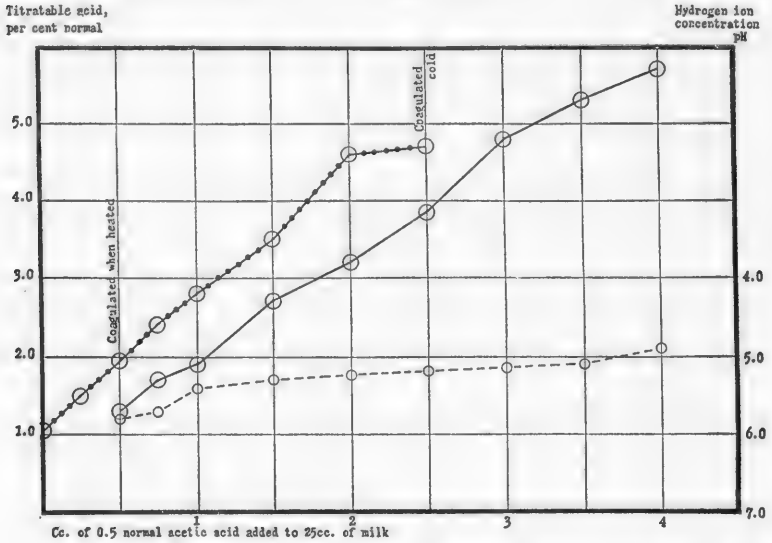
TEXT-FIG. 2. Titratable acidity and hydrogen ion concentration of fat-free milk after inoculation with *B. pyogenes*.

Seven tubes of fat-free milk were inoculated with *Bacillus pyogenes*. A tube was withdrawn for titration of total acidity and determination of hydrogen ion concentration[4] after incubation for 1, 2, 3, 4, 6, 9, and 15 days. The results are plotted in Text-fig. 2. The titratable acidity increased steadily. The hydrogen ion concentration of the whey

[4] Determinations of hydrogen ion concentration were made by the colorimetric method of Clark and Lubs (1917, *a*).

increased very little after the 6th day and reached a maximum of pH 5.6 in 15 days. The milk showed visible coagulation without application of heat on the 2nd day of incubation when the hydrogen ion concentration of the whey was pH 6.2 and the titratable acidity of the curdled milk was 1.7 per cent normal.

For comparison with these results tubes of milk were acidified with increasing amounts of 0.5 N acetic and hydrochloric acids. The results of titration of total acidity and determination of hydrogen ion concentration are plotted in Text-figs. 3 and 4.

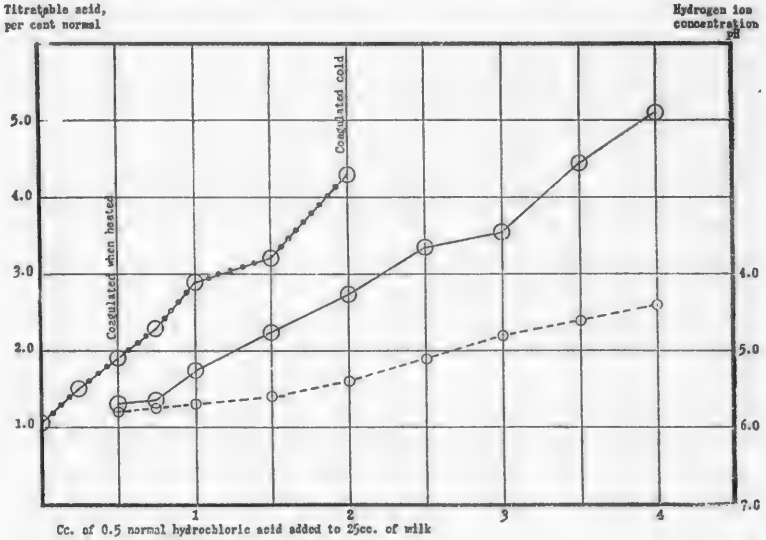


TEXT-FIG. 3. Total acidity and hydrogen ion concentration of fat-free milk after the tubes had been acidified with increasing amounts of acetic acid.

As was to be expected the hydrogen ion concentration showed more rapid increase with addition of hydrochloric acid than with similar amounts of acetic acid. With both the strong and the weak acid, however, the milk was coagulated without application of heat when it had reached a titratable acidity of 4.3 and 4.7 per cent normal respectively and the whey had a hydrogen ion concentration of pH 5.4 to 5.2. As was also to be expected the titratable acidity of the weak acid accompanying a hydrogen ion concentration sufficient to coagu-

late the milk was higher than that of the strong acid, the latter being more highly ionized. The difference is even more apparent in the titratable acidity of the whey.

In fermented bouillon plus 0.2 per cent of calcium chloride and 2.0 per cent of sodium caseinate *Bacillus pyogenes* produced a casein coagulum or sediment within 24 hours at 37°C. In the absence of calcium chloride no coagulation occurred. The same media gave similar results with rennet. These tests were made in media of four different reactions; namely, pH 6.2, 6.8, 7.1, and 7.7. After inoculation



TEXT-FIG. 4. Total acidity and hydrogen ion concentration of fat-free milk after the tubes had been acidified with increasing amounts of hydrochloric acid.

with *Bacillus pyogenes* and incubation for 20 hours, during which time coagulation occurred in all of those containing calcium chloride, the reactions were pH 6.2, 6.5, 6.7, and 7.2 respectively. The sedimented coagulum in the last tube was the most voluminous and the supernatant fluid from this tube gave no further precipitate when acidified with acetic acid. In the tube with pH 6.2 the sediment was more compact than in the others but acidification of the supernatant fluid showed that some casein was still in solution. It has also been found

that when *Bacillus pyogenes* is grown in fermented bouillon, plain bouillon, serum bouillon, dextrose bouillon, or dextrose serum bouillon plus sodium caseinate, the medium becomes very cloudy, almost opaque, in 24 hours and then clear again in another day or two. No sediment is formed or at most only a sediment of the organism itself. When the medium clears, however, the casein has disappeared and cannot be precipitated by acetic or nitric acid. Serum does not completely disappear and can be precipitated by nitric acid in cultures more than a week old. Apparently in the dextrose bouillon the casein is digested before the acidity rises sufficiently to precipitate it.

The preparation of casein calcium bouillon is attended with some difficulty. The method employed was as follows: To the sterile fermented bouillon add sufficient sterile 10 per cent calcium chloride to make a 0.2 per cent solution. Add sufficient sterile hydrochloric acid to dissolve the calcium phosphate which has been precipitated. At this point the medium reacts pH 6.2 to 6.4. Add sufficient 10 or 20 per cent sterile casein solution to make 2.0 per cent of casein in the bouillon. After the casein has been added the medium may be made alkaline if desired by the addition of sterile sodium hydrate. The calcium is apparently held by the casein so that it is not again precipitated as phosphate in the presence of a moderate amount of alkali. The preparation of this medium could probably be simplified by the use of calcium caseinate rather than sodium caseinate.

Since milk is coagulated by *Bacillus pyogenes* at a titratable acidity far below that required of hydrochloric or acetic acid and also at a much lower hydrogen ion concentration, and since soluble casein is coagulated in a neutral or slightly alkaline sugar-free medium, it appears that the coagulation of milk by *Bacillus pyogenes* is an enzyme rather than an acid coagulation. It is also to be noted that the casein is digested even in the presence of an excess of fermentable sugar. The process of digestion, however, seems to stop short of ammonia production since in a milk culture incubated for 3 weeks no increase in ammonia could be detected by Folin's method.

Immunological Study.

Four representative strains of *Bacillus pyogenes* were selected for the immunization of rabbits. Strain IV was selected because it showed slight morphological differences from the others. Strains VII, VIII, and X were selected because they were isolated from different lesions; *i.e.*, lungs, uterus, and a fetus, respectively. Rabbits

TABLE II.

Cross-Precipitation of Strains IV, VII, VIII, and X.

Strain No.	Serum No.	Dilutions.					Controls.	
		1:10	1:20	1:40	1:80	1:160	Normal rabbit serum, 1:10.	Salt solution.
IV	IV	++++	+++	++	++	+	−	−
	VII	++++	++	+	−	−	−	−
	VIII	++	Sl.	−	−	−	−	−
	X	++++	++	+	−	−	−	−
VII	IV	++	++	+	Sl.	−	−	−
	VII	++++	++++	++	−	−	−	−
	VIII	+	−	−	−	−	−	−
	X	++++	++	+	Sl.	−	−	−
VIII	IV	++	+	Sl.	−	−	−	−
	VII	++++	+++	+	Sl.	−	−	−
	VIII	++	+	+	+	Sl.	−	−
	X	+++	++	++	+	+	−	−
X	IV	++	+	Sl.	−	−	−	−
	VII	+++	++	+	+	−	−	−
	VIII	+	+	Sl.	−	−	−	−
	X	++++	+++	++	+	−	−	−

In the tables ++++ indicates maximum precipitation; Sl., slight precipitation.

were given at first several series of subcutaneous and intravenous injections of killed cultures, and later increasing amounts of living bouillon cultures intravenously at intervals of about 1 week. The rabbits tolerated the injections well and though all eventually succumbed to *Bacillus pyogenes* infection noticeable lesions did not develop until after the immune serum had been secured.

TABLE III.

Precipitation of All Strains by Sera IV and X.

Strain No.	Serum No.	Dilutions.					Controls.	
		1:10	1:20	1:40	1:80	1:160	Normal rabbit serum, 1:10.	Salt solution.
I	IV	++	+	Sl.	−	+	−	−
	X	+++	++	++	+	Sl.	−	−
II	IV	+++	+++	+	Sl.	−	−	−
	X	++++	+++	++	+	+	−	−
III	IV	++	+	+	Sl.	−	−	−
	X	++++	++	+	−	−	−	−
IV	IV	++++	+++	++	++	+	−	−
	X	++++	++	+	−	−	−	−
V	IV	++	++	+	−	−	−	−
	X	++++	+++	++	±	−	−	−
VI	IV	+++	++	+	−	−	−	−
	X	++++	++++	+++	++	+	−	−
VII	IV	++	++	+	Sl.	−	−	−
	X	++++	++	+	"	−	−	−
VIII	IV	++	+	Sl.	−	−	−	−
	X	+++	++	++	+	+	−	−
IX	IV	++	+	Sl.	−	−	−	−
	X	+++	++	+	−	−	−	−
X	IV	++	+	Sl.	−	−	−	−
	X	++++	+++	++	+	−	−	−
XI	IV	+	Sl.	−	−	−	−	−
	X	++++	++	+	−	−	−	−
XII	IV	++	++	+	Sl.	−	−	−
	X	++++	++++	+++	++	+	−	−

The titration of agglutinins was unsatisfactory because all but one or two strains were agglutinated to a considerable extent by normal rabbit serum in dilutions of 1:100 or 1:1,000. There was relatively little agglutination in salt solution controls. By regarding as positive only those tubes in which agglutination was stronger than in the normal serum controls it was evident that the immune sera produced agglutination of the homologous strains and many others in dilutions of 1:800 to 1:3,200. However, the normal agglutinins were such a disturbing factor that it is considered unsafe to draw any conclusions as to the relationship of the various strains on the basis of agglutination.

More satisfactory results have been obtained by titrating the precipitins. The precipitinogen used in the titrations consisted of the clear supernatant fluid obtained by centrifuging bouillon and blood bouillon cultures after incubation for 1 month. During incubation the cultures were frequently shaken. The blood bouillon and the plain bouillon yielded equally good precipitinogen. The precipitin titer of the sera was much lower than the agglutination titer but there was no precipitation in the controls and the results were quite definite. In Table II are given the results of cross-precipitation of the four strains employed for immunization of rabbits. There is precipitation of all the strains by each of the sera. Except in the case of Strain VIII the precipitinogen of each strain is precipitated best by the homologous serum. Serum VIII was rather a weak serum probably due to the fact that the rabbit from which it was obtained could not be injected regularly because of its having "snuffles." No great diversity among the strains is revealed by the precipitin titration. Strain IV, however, does seem to stand a little apart from the other three. Serum IV and Serum X were therefore selected for titration with all the strains. The results are recorded in Table III. The precipitinogens of all the strains except No. IV show greater precipitation with Serum X than with Serum IV. It appears that Strain IV is slightly different from the others immunologically as well as morphologically.

Pathogenicity for Rabbits.

According to Berger (1908), Holth (1908), and others, rabbits are the most susceptible of the small laboratory animals to experimental infection with *Bacillus pyogenes*. Guinea pigs are less susceptible, and mice least so.

In addition to the four rabbits used for immunization five others were injected intravenously with a single dose of 3 or 4 cc. of living bouillon culture, two with Strain IV and three with Strain X. Those immunized against Strains IV, VII, VIII, and X were repeatedly injected intravenously with these strains. Eight of the rabbits succumbed to the infection or were killed when in a badly crippled or moribund condition. The ninth may have died as a result of bleeding from the heart. There were no noticeable immediate symptoms following injections, no toxic symptoms, but usually a rise in temperature within 48 hours. Following this the rabbits appeared normal for 2 or 3 weeks. The first symptom of infection was often a progressive loss in weight followed by lameness or in four cases by paralysis. One of the paralyzed rabbits died during our absence from the laboratory and a complete autopsy was not obtained. The lesions found in the remaining seven rabbits may be classified as follows:

Lesions of bones in six rabbits (vertebræ three, femur four, rib one, tibia one).
Lesion of joint in one rabbit.
Lesions of muscles or tendons in three rabbits.
Lesion of lymph node in one rabbit.
Endocarditis in one rabbit.
Pneumonia in one rabbit.
Kidney abscesses in one rabbit.

All the above lesions were studied histologically and culturally, and were found to be due to the organism injected. A summary of these results is given in Table IV.

Rabbit J was injected subcutaneously only with bouillon cultures of Strain X at three different times as follows:

1st day. Injection A, 0.2 cc.; Injection B, 1.0 cc.
7th day. Injection C, 0.2 cc.; Injection D, 0.4 cc.; Injection E, 0.6 cc.
10th day. Injection F, 0.2 cc.; Injection G, 0.4 cc.; Injection H, 0.6 cc.
12th day. Rabbit chloroformed. Abscesses removed and fixed in Zenker's fluid. The rabbit had lost about 100 gm. in weight but was otherwise apparently well. At autopsy the subcutaneous lesions were the only ones found.

The abscesses at the time of removal were therefore 2, 5, and 11 days old. The youngest abscesses appeared grossly as flat discs about the size of five cent pieces and on gross section appeared to be composed of a fibrous tissue infiltrated with a small amount of yellowish

translucent viscid pus, and surrounded by soft hemorrhagic edematous tissue. The largest and one of the oldest abscesses, B, was firm and nodular, about 1 cm. in diameter, composed of a thick capsule of dense fibrous tissue enclosing a thick creamy yellow somewhat viscid pus.

A stained section of one of the youngest abscesses, H (Fig. 10), is roughly oval, about 7 mm. long by 2 mm. broad. It lies in a loose areolar connective tissue with a layer of transversely cut muscle fibers within 1 mm. of one side of the abscess. There is no definite capsule. The abscess is bordered by a thin layer of fibrin and necrotic connective tissue. The connective tissue on all sides is infiltrated by polymorphonuclear leucocytes while the perimysium of the adjacent muscular tissue contains many eosinophilic cells. There are also some large mononuclear cells many of which are doubtless fibroblasts though some may be endothelial leucocytes. Some of the muscle fibers nearest the abscess are invaded by polymorphonuclear leucocytes and are undergoing heterolysis. The contents of the abscess are principally a mass of degenerating polymorphonuclear leucocytes. Scattered about are bits of collagenic fibers, each embedded in a mass of *B. pyogenes* as though the bacilli are growing on the collagenic fibers as a medium (Fig. 14). Very few bacilli are found elsewhere than clustered about these fibers. About these individual masses of bacilli there is always an area of compact necrosed cells with few visible nuclei and in one part these areas by confluence have formed the beginning of the characteristic central zone of the older abscesses.

The older abscesses, A and B, are spherical and surrounded by dense fibrous capsules, 2 to 3 mm. thick (Fig. 11). The abscess is differentiated into three fairly distinct zones. The central zone (Fig. 11, *a*) is a ragged granular mass of dead and disintegrating cells with few visible nuclei. Within this zone many bacilli are scattered about, but towards its periphery these are in masses only a few of which still contain a fragment of collagenic fiber. Apparently these fibers are digested by the proteolytic action of the bacilli. Outside the central zone no bacilli are found. In the second or intermediate zone (Fig. 11, *b*) most of the cells have deeply stained nuclei which, however, exhibit pycnosis, caryorrhexis, or caryolysis. The outer zone (Fig. 11, *c*) resembles the central zone in general appearance. There are few visible nuclei, but no bacilli. It is bordered, however, by large mononuclear macrophages laden with nuclear and other cell debris.

In sections the bacilli are best stained by Gram's method used according to Holth's (1908) directions. Stirling's aniline gentian violet containing 1 or 2 per cent by weight of the dry stain has given us excellent results. To show the association of bacilli with collagenic fibers we have obtained excellent preparations with the following stains.

Orth's lithium-carmine, 20 minutes.

Acid alcohol, 3 minutes.

Stirling's aniline gentian violet, 10 seconds.

TABLE IV.

Rabbits Inoculated with B. pyogenes.

Rabbit.	Sex.	Strain No. Injection.	Maximum temperature.	Weight variation.	Localizations.	Result.
			°C.	gm.		
A	M.	IV. Repeated subcutaneous and intravenous injections for immunization.	40	1,890–2,355	Yellow spots in liver; hemorrhagic foci in lungs. (Incomplete autopsy.)	Died suddenly during night after being bled, 39th day after first injection of living culture.
B	"	VII. Repeated subcutaneous and intravenous injections for immunization.	41	1,650–1,865–1,380	Left femur; both kidneys; heart valve; epicardium.	Died after period of weakness 97th day after first injection of living culture.
C	"	VIII. Repeated subcutaneous and intravenous injections for immunization.	40.8	1,850–1,944–1,705	Muscle abscess in left quadriceps extensor femoris muscle. (Recurrent attacks of snuffles.)	Chloroformed 4½ mos. after first injection of living culture.
D	"	X. Repeated subcutaneous and intravenous injections for immunization.	40.2	1,650–2,010	3rd and 4th lumbar vertebræ; abscess surrounding and invading rib.	Paralysis of both hind legs. Chloroformed 5 mos. after first injection of living culture.
E	"	IV. 3 cc. intravenously.	39.8	1,960–2,125–1,945	(Incomplete autopsy.)	Paralysis of both hind legs and bladder. Died suddenly on 34th day after injection.

F	F.	X. 3 cc. intravenously.	41.2	1,935–1,980–1,040	Left femur.	Died 27 days after injection.
G	M.	IV. 4 " "	40.5	1,645–1,935–1,470	1st thoracic vertebra; right knee; both shoulder joints (periarticular abscesses).	Paralysis of left hind leg. Chloroformed on 81st day after injection.
H	"	X. 4 " "	41.4	1,835–1,895–1,240	Right femur; tendon of left gastrocnemius muscle; left instep; lungs.	Died on 13th day after injection.
I	"	X. 3 " "	39.8	1,815–1,910–1,240	1st, 2nd, and 3rd lumbar vertebræ; crest of right tibia; trochanter and gluteus minimus muscle of right femur; right zygomatic fossa; right biceps brachii muscle; left cubital gland.	Paralysis of both hind legs. Chloroformed on 64th day after injection.
J	"	X. Multiple subcutaneous injections.	39.4	3,580–3,415	Subcutaneous abscesses at points of injection.	Chloroformed 11th day after first injection.

Wash in water.
Lugol's iodine solution, 30 seconds.
Wash in water. Blot.
Decolorize in absolute alcohol.
Wash in water.
Mallory's aniline blue and orange G, 20 minutes.
Wash in water.
Decolorize and dehydrate in 95 per cent and absolute alcohol.
Xylol. Mount in balsam.

By the above method *B. pyogenes* is stained purple, collagenic fibers bright blue, leucocytes and tissue cell nuclei red or deep orange, blood, fibrin, and muscle yellow.

Of the lesions resulting from the intravenous injection of cultures a few deserve brief description. The most frequently produced lesions were those of the bones. Such lesions have been reported by Berger (1908), Holth (1908), and Koske (1906). Their protocols show that they encountered paralyses also in experimentally infected animals. Koske, unable to find lesions in the cords of paralyzed pigs, considered it possible that a specific neurotoxin might be involved but was unable to obtain toxic effects with filtered cultures. He does not mention examining the vertebræ of these animals. Four times we encountered paralyses in injected rabbits. In the three that were thoroughly autopsied were found lesions on the ventral floor of the spinal canal. These lesions were not visible from the ventral side of the spinal column and in fact could not be found until the cord had been removed. There were then found abscesses exerting pressure against the ventral side of the cord. In no case was the dura penetrated nor were the meninges infected. In the case of Rabbit G an abscess lay between the dura and the vertebral periosteum without invading the body of the vertebra. In Rabbits D and I were found intervertebral abscesses obliterating the intervertebral cartilages, invading the bodies of the vertebræ, and eroding the bone with more or less destruction of the floor of the spinal canal (Fig. 12).

In a stained section there is found proliferation of connective tissue about the abscess. The center of the focus is composed of a mass of disintegrated cells and nuclei which appear to have been small mononuclear cells rather than polymorphonuclear leucocytes, differing in this respect from the abscesses in the subcutis and other soft parts. In the proliferating fibrous tissue bordering and sometimes sur-

rounding the abscesses in the vertebræ are many large mononuclear cells (Fig. 15). Stained by Gram's method many of these large cells are seen to be filled with *B. pyogenes* (Fig. 16). Holth regards these cells as young fibroblasts rather than endothelial cells. In the necrotic center of the abscess bacilli lie scattered about.

The lesion here described is much like the "*grösseren Knoten*" described by Holth and found by him in various parts of the body—lungs, subcutis, peritoneum, etc. He describes masses of "*Rundzellen*" in the center of the abscess undergoing necrosis and disintegration, an intermediate zone of tissue resembling the round cell masses of smaller abscesses, and an outer capsule of connective tissue interrupted by small masses of round cells. As the tumor grows the round cell masses fuse and the process of disintegration advances. He then describes the bacilli scattered about in the center of the abscess and within what he regards as large connective tissue cells of the capsule. He does not mention the presence of polymorphonuclear leucocytes in the abscess, whereas we found them to predominate in abscesses of soft parts. It is to be noted, however, that because of pycnosis these cells often resemble round cells. It is to be noted also that in the subcutaneous abscesses described above no bacilli were found in the cells of the capsule, possibly because the abscesses studied by us were not old enough.

Lesions in the long bones—femur and tibia (Fig. 13)—were fundamentally like those in the bodies of the vertebræ, modified by the tissues encountered. The predominating cells of the reaction resembled plasma cells. In places there was marked proliferation of connective tissue. In places the bacilli appeared to grow freely among the cells of the bone marrow. Occasionally bone lacunæ were seen filled with masses of bacilli. There was always erosion of the bone making it quite porous. In two cases the femur broke under very little strain as the rabbits were being tied out for autopsy. A nodular encapsulated abscess enveloped the rib of Rabbit D eroding it from without. The abscess may have been subperiosteal in origin.

The knee of Rabbit G contained a glairy viscid pus and one of the joint surfaces was eroded.

In Rabbits C, H, and I true myositis and tendinitis were encountered. In these cases the muscle fibers had been completely heterolyzed or digested and the muscle converted into a closed sac of viscid

glairy pus within the epimysium (Fig. 9). There were present in the pus and perimysium plasma cells, endothelial leucocytes, neutrophilic polymorphonuclear leucocytes, and, especially conspicuous, eosinophilic leucocytes. Bacilli were abundant in all but the oldest lesions.

The diseased mitral valve of Rabbit B was encrusted with masses of *Bacillus pyogenes*, leucocytes, and necrotic tissue. The tissue of the valve was largely fibrin.

In the pneumonic lung of Rabbit H masses of bacilli were found beneath the pulmonary pleura. It appeared that growth had started from bacilli lodged in the pleural capillaries. The alveoli were filled with blood, fibrin, and desquamated epithelium.

The kidney abscesses of Rabbit B were in the form of pyramids with bases at the cortex. Around the borders of the abscesses the glomeruli and the capillaries of the interstitial tissue were plugged with leucocytes and masses of bacilli. The interlobular arteries were similarly plugged.

Resemblance to Other Organisms. Classification.

The close resemblance of *Bacillus pyogenes* to streptococci has been mentioned above. This is especially true of some strains when grown on certain media, a resemblance so close that the bacteriologist working with milk or animal diseases must be on his guard not to confuse them. *Bacillus pyogenes* produces laking of a suspension of blood or blood corpuscles in salt solution as do the hemolytic streptococci of human origin (Brown, 1920). Its limiting hydrogen ion concentration in dextrose bouillon would also mislead one to place it among the human streptococci (Avery and Cullen, 1919). On the other hand, *Bacillus pyogenes* liquefies coagulated blood serum and is distinctly diphtheroid at times. Morphologically, therefore, it may be one of the diphthero-streptococci now and then described. Such organisms, are occasionally isolated from human lesions, especially pneumonias and though *Bacillus pyogenes* has never been identified in man, it would be well for bacteriologists to keep it in mind. Since it is found in several species of animals and is widely disseminated in milk it may be that there are rare cases of human infection.

There is also found in animals a group of small Gram-positive organisms closely resembling *Bacillus pyogenes* morphologically and culturally. We have studied two such strains from the livers of calves and one from the lung of a hog. The strains from calves were apparently alike, and had the same fermentation reactions as *Bacillus pyogenes*. They were cultivated with greater difficulty than the latter, however. They grew best under partially anaerobic conditions and would not grow in serum-free media. There was no apparent growth in milk. Gelatin and coagulated serum were not liquefied and growth on the latter was scarcely visible. There was very little laking of horse blood in agar plates. A rabbit was repeatedly injected intravenously with several cubic centimeters of serum bouillon cultures of one of these strains with no ill effects. Serum from this rabbit precipitated precipitinogens of both of these strains in dilution of 1:80 or 1:160 but produced no precipitation of the precipitinogens of strains of *Bacillus pyogenes*. Neither did *Bacillus pyogenes* antisera precipitate precipitinogens of these two strains.

A strain from a hog also resembled *Bacillus pyogenes* morphologically. It differed from *Bacillus pyogenes* and the other two strains just described in that it fermented salicin. It resembled the two strains from calves in failing to liquefy gelatin or coagulated serum but grew much better than those strains. No visible change was produced in milk but sufficient acid was produced so that when the culture tube was placed in boiling water the milk was coagulated. An indefinite zone of hemolysis was produced in the blood agar plate.

Glage (1913) points out a certain resemblance of *Bacillus pyogenes* to the bacillus of swine erysipelas. Morphology, growth in gelatin, and the lesions produced in hogs and small experimental animals serve to differentiate the two.

Dunkel (1908) thought that *Bacillus pyogenes* could be transformed into *Bacillus pseudotuberculosis ovis* by animal passage and therefore regarded the two as of the same species. The two are certainly not alike when studied as isolated from their respective hosts. The dry colonies described as characteristic of *Bacillus pseudotuberculosis*, pigmented colonies on coagulated blood serum, and failure to produce change in milk are not at all like *Bacillus pyogenes*. There may be some morphological resemblance.

Priewe (1911) and Glage (1913) have claimed that *Bacillus pyogenes* belongs to the influenza bacillus group for the following reasons: hemoglobinophilic habit, form, size, non-motility, lack of spores, abundance in green pus, growth at high temperature only, and slight virulence for small laboratory animals. Priewe claimed that a *Bacillus pyogenes* antiserum agglutinated *Bacillus influenzæ*. We have not tried to repeat the latter observation. However, we do regard it as well established that *Bacillus pyogenes* is Gram-positive and that it is not hemoglobinophilic. The other characters enumerated by the above authors are common to so many and dissimilar organisms that they are of little value as evidence of relationship of these two organisms. *Bacillus pyogenes* does not have the foul odor characteristic of *Bacillus influenzæ* and the two do not produce similar lesions in rabbits. Grips (1903), Grips, Glage, and Nieberle (1904), and Priewe (1911) have regarded swine-plague as due primarily to *Bacillus pyogenes* with *Bacillus suisepticus* as a secondary invader. They regard the primary infection as a "*Tierinfluenza*." The views of these authors have not been accepted by others. Olt (1904) has criticized them thoroughly but asserts that many of the lesions characteristic of chronic swine-plague are due to *Bacillus pyogenes*.

The forms assumed by *Bacillus pyogenes* bear a striking resemblance to those of *Asterococcus mycoides* described by Borrel, Dujardin-Beaumetz, Jeantet, and Jouan (1910) as the cause of bovine pleuropneumonia. The latter organism is, however, much the smaller. The work of the authors mentioned is almost wholly morphological and the result of staining by Gram's method is not mentioned. Buchanan (1918), however, places *Asterococcus* in the Gram-negative subtribe Hemophilinæ. The other genus of this subtribe is *Hemophilus*, of which the type species is *Hemophilus influenzæ*, the influenza bacillus of Pfeiffer. Since Buchanan and Murray (1916) describe *Bacillus pyogenes* as a member of the hemophilic or influenza group they presumably classify it also in the subtribe Hemophilinæ where it can hardly belong in view of its being Gram-positive. Preisz (1906) regarded *Bacillus pyogenes* as one of the "*Corynebakterien*." We are more inclined to place it in the genus *Corynebacterium* as defined in the Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types (1917) than in the so called influenza group.

SUMMARY.

Bacillus pyogenes is probably quite common in this country, as it is known to be in Europe.

A careful study of twelve strains from cattle and one from a hog has disclosed the following characteristics which have not been reported or have been in dispute.

Bacillus pyogenes is Gram-positive and pleomorphic, producing forms ranging from short chains of streptococcoid elements to branching filaments.

It is hemolytic, producing the beta type of hemolysis in blood agar. It is not hemoglobinophilic, though its growth is greatly favored by some higher protein material such as egg albumin, serum, or blood.

It ferments xylose in addition to the substances previously reported.

The coagulation of milk by *Bacillus pyogenes* is primarily an enzyme coagulation and the subsequent digestion of the curd takes place in an acid medium.

The intravenous injection of rabbits was invariably fatal. The lesions most commonly developed were those of the bones. Paralysis was frequently produced, and in each case was caused by lesions in the vertebræ exerting pressure against the ventral columns of the spinal cord. Muscle abscesses were also frequently produced.

The authors regard the organism as belonging to the *Corynebacteria* rather than to the influenza group.

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EXPLANATION OF PLATES.

PLATE 14.

FIG. 1. A horse blood agar plate culture of *B. pyogenes* X after incubation for 48 hours. The large round hemolyzed area was produced by a few particles of saponin.

PLATE 15.

Gram stains. Magnification × 1,000.

FIG. 2. The bacillary form of Strain X.

FIG. 3. The fusiform form of Strain IV.

FIG. 4. The fusiform form of Strain X.

FIG. 5. The filamentous or branching form of Strain IV.

FIG. 6. The diphtheroid form of Strain X showing a few buds.

FIG. 7. The streptococcoid form of Strain IV.

FIG. 8. The streptococcoid form of Strain X.

PLATE 16.

FIG. 9. Rabbit H. Abscess in tendinous end of gastrocnemius muscle at *a*. Above the abscess is a section of the distal end of the flexor digitalis pedis sublimis muscle. Eosin and methylene blue stain. × 10.

FIG. 10. Rabbit J. Subcutaneous abscess H, 48 hours after injection. The capsule and zonal arrangement of the abscess not yet developed. Gram and aniline blue stain. × 5.

FIG. 11. Rabbit J. Subcutaneous abscess A, 11 days after injection. A very thick fibrous capsule (*d*) about the abscess and the three zones (*a*, *b*, *c*) of the abscess described in the text are shown. Eosin and methylene blue stain. × 5.

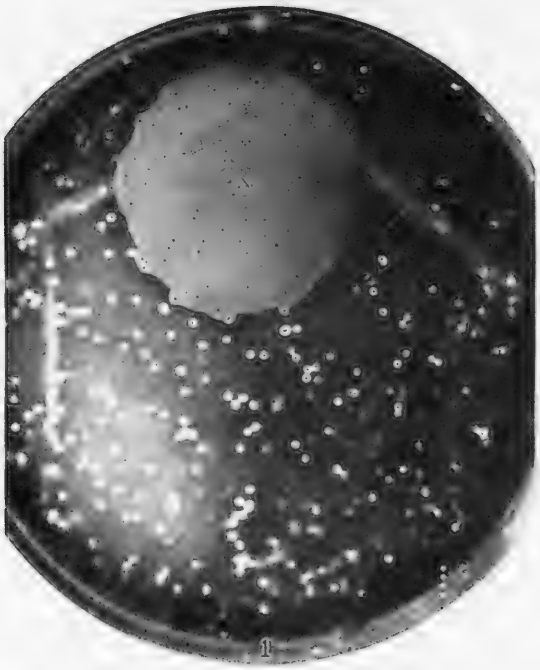
FIG. 12. Rabbit D. Abscess occupying the body of the third lumbar vertebra at *a*, the spinous process and dorsum of the spinal foramen having been removed. Only a small bony fragment of the floor of the spinal canal remains at *c*. Eosin and methylene blue stain. × 5.

FIG. 13. Rabbit I. Abscess in crest of tibia at *a*. Transverse section. Eosin and methylene blue stain. × 10.

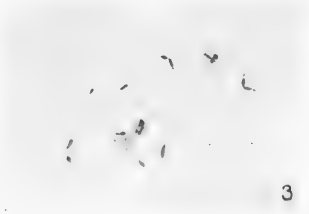
FIG. 14. Rabbit J. A field from the center of the abscess shown in Fig. 10, showing the minute bacilli clustered about remnants of connective tissue fibers. Gram and aniline blue stain. × 430.

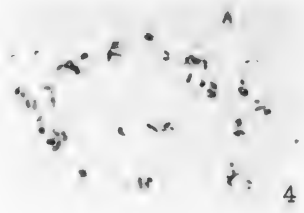
FIG. 15. Rabbit D. A field from the region marked *b* in Fig. 12, showing large mononuclear cells probably on the border of an abscess. Eosin and methylene blue stain. × 430.

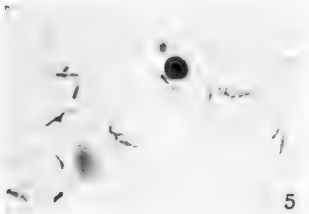
FIG. 16. Rabbit D. A field from the same region as Fig. 15, showing the large mononuclear cells filled with *B. pyogenes*. Gram stain. × 430.

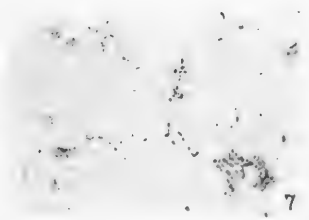


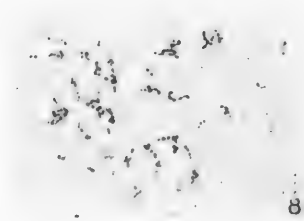
(Brown and Orcutt: *Bacillus pyogenes.*)











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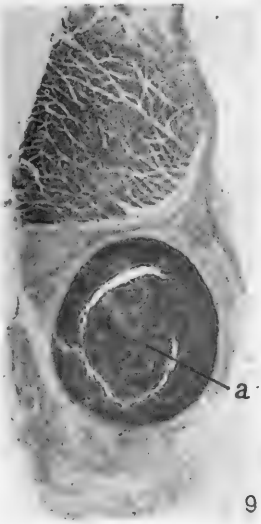

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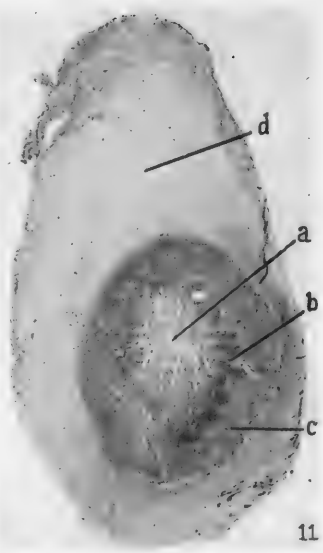

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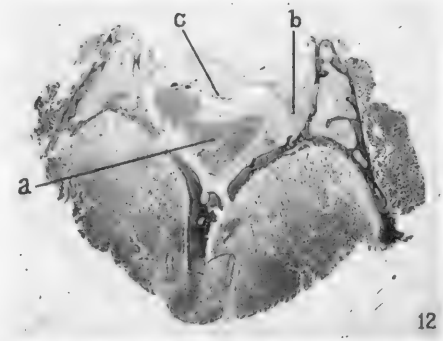

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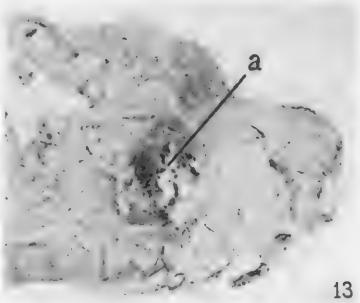

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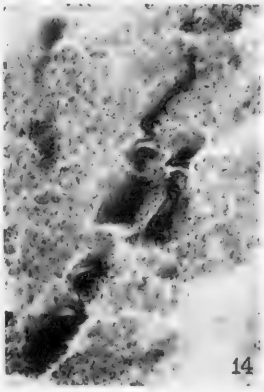

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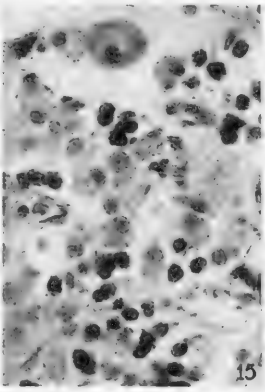

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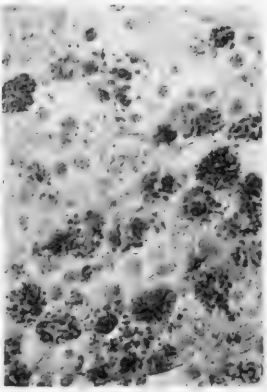

16

(Brown and Orcutt: *Bacillus pyogenes*.)

THE BILIARY FACTOR IN LIVER LESIONS.

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PLATES 17 TO 22.

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The qualification "biliary" has long been applied to a diverse group of hepatic lesions distinguished by a peculiar cirrhosis with more or less evidence of stasis in the finer bile ducts and inflammation of their walls. The actual part played by bile in the production of the connective tissue changes here seen and of chronic lesions in general of the human liver is not definitely known. According to some authors human bile is incapable of causing any permanent hepatic injury. If this be true man differs from all other well studied animals, for in them without exception aseptic bile stasis results in important local changes. There is positive evidence, too, that human bile may on occasion be the cause of important damage to the liver. Sudden total obstruction of the common duct results in the so called icteric necroses,[1] which may attain to the size and character of "biliary infarcts,"[2] large wedge-shaped areas of bile-stained necrosis such as regularly follow obstruction in rabbits and guinea pigs. Long continued stasis, when uncomplicated by infection, as is rarely the case, has been known to result in stellate cirrhosis with many new-formed bile ducts, a lesion found in the rabbit under like circumstances;[3] and, as in this animal, complete local stasis leads to liver atrophy.[4] Further examples might be cited. For the purposes of the present work we shall assume that human bile, while innocuous as compared with that of certain other species, can produce liver injury. Our

[1] Eppinger, H., *Beitr. path. Anat. u. allg. Path.*, 1902, xxxi, 230.

[2] Carnot, P., and Hervier, P., *Arch. méd. exp. et anat. path.*, 1907, xix, 76.

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[4] Brissaud, E., and Sabourin, C., *Arch. physiol. norm. et path.*, 1884, iii, series 3, 345.

aim has been to obtain through experiment a better understanding of "biliary" lesions with special reference to the share of bile in their causation. No attempt will be made to ignore the factor of infection, which without doubt has for many instances a prime importance.

Choice of Animals.

The differences in the commoner laboratory animals as regards amount of the bile and its local effects have been admirably set forth by Quincke and Hoppe-Seyler.[5] The years since their summary have added little that is new. The rabbit and guinea pig respectively secrete nearly seven and eight times as much bile as the dog, and ten and twelve times as much as the cat. Man's rate of secretion places him near to the last mentioned animals. In them the constitutional effects of total biliary obstruction are more marked than the local, while in man death usually occurs of toxemia before cirrhotic changes are manifest.[3] In the guinea pig and rabbit, on the contrary, closure of the common duct results in fulminant liver destruction with cirrhosis, and the death of the animal, which follows after a few weeks at most, is traceable to this cause. The development of lesions following local duct obstruction is far more rapid than in the dog and cat, changes that may take a twelve-month in these latter[6] requiring only a fortnight or less in the rabbit. And in the rabbit, furthermore, secondary infection occurs but rarely, whereas in the dog it is so frequent a confusing factor as to have balked many investigators. On the basis of recent experience we have no hesitation in asserting that had dogs instead of rabbits been employed for the present work years instead of months would have been required to obtain an understanding of the simpler principles ruling biliary lesions—principles that are of evident general application, and to be derived as surely from observations upon one mammalian species as another.

[5] Quincke, H., and Hoppe-Seyler, G., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 59.

[6] Harley, V., and Barratt, W., *J. Path. and Bacteriol.*, 1901, vii, 203.

Method.

In work already reported,[7] we have taken advantage of the singular fitness of rabbits for experiments upon the liver which in this species consists of two well separated masses, each with its own bile duct and blood vessels. The smaller, or lobe mass, as it may be called, forming approximately one-quarter of the whole —23.7 per cent after ablation of the caudate lobe (4 per cent)—will accommodate on emergency the entire portal stream and maintain the animal in good health when the larger mass, or main liver (72.3 per cent), is ablated. The liver of the rabbit adapts itself to new conditions far more rapidly than that of the dog or cat. Thus, for example, the gradual parenchymal atrophy and hypertrophy which follow local portal diversion require but 2 months in the rabbit for their approximate completion, whereas in the dog several times this period is required.[7] There exists, however, a very considerable drawback to the clinical study of jaundice in the rabbit in the fact that the bile pigment of the animal reacts but poorly to the ordinary color tests. Our work has not been of a sort to make this difficulty felt. A second and serious potential objection to rabbits is to be found in the intercurrent hepatic cirrhosis prevalent in certain localities. The lesion has been rare in our experience as in that of some others,[8] and individuals showing it at operation, the first step in every experiment, have been discarded. As a further control to the condition of the liver, the caudate lobe has regularly been ablated for section. The total bulk of the organ is thus reduced by about 4 per cent, as already stated.

The rabbits weighed from 1,400 to 2,500 gm. The general method of operation has been described in another connection.[7] Bile ducts to be ligated were dissected free of the surrounding structures and tied twice with fine black silk, but not cut, since this precaution against a restoration of continuity was found unnecessary. The ducts are extremely delicate. Almost all the postoperative mortality was due to rupture of them above the ligature. When a branch of the portal vein was to be tied, it was dissected out. Special care was always taken to avoid injury to the hepatic artery, for this in the rabbit usually results in more or less widespread necrosis. Recovery from operation was in general uneventful. For the better analysis of the lesions most of the animals were killed early, with chloroform or by bleeding from the heart. Bits of the liver tissue were placed upon agar and in bouillon, and additional stroke and loop inoculations were made. Infection was rare, and instances showing it were ruled from consideration. A scattered, localized coccidiosis was fairly frequent, however. It had no evident complicating effect upon the liver changes.

Zenker's fluid, formaldehyde, and alcohol were used as fixatives.

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[7] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609. All operations were performed under ether anesthesia.

[8] Grover, A. L., *J. Am. Med. Assn.*, 1915, lxiv, 1487.

The amount of the bile and the pressure developing within obstructed ducts are both relatively great in well fed animals.[9] To this circumstance and to differences in the character of the food[10] are probably attributable most of the considerable quantitative variations that may be observed in lesions resulting from a single procedure. Our animals were not fed for 18 hours prior to operation, but immediately thereafter they were placed upon the mixed diet of normal rabbits.

Results of Total Obstruction.

Nearly all attempts to obtain by experiment an understanding of biliary lesions, from Leyden's time on, have centered about the production of total stasis by ligation of the common duct; and there exist many descriptions of the ensuing changes.[11] Our observations have but confirmed the general findings, while yielding an interpretation of them that has proven productive.

The rabbit secretes bile so copiously—136.8 gm. per kilo of animal in 24 hours, according to Heidenhain[10]—that obstruction of the common duct is followed practically at once by marked distension of the passages. Within a few hours bile-stained parenchymal necroses appear, of all sizes, from those that involve but a single cell to wedge-shaped areas, the so called biliary infarcts, 0.5 cm. or more in diameter. The larger ones are stained a bright yellow-green and frequently show hemorrhages. All are obviously the result of direct extravasation of the bile, either within the lobule or at its periphery where the smallest collecting ducts have ruptured. An identical rupture at this latter point, the weakest one in the collecting system, occurs on the injection under pressure of fluid into the common duct.

Within a few days dilatation of the larger ducts becomes great, and pericholangitis develops, followed soon by a spreading, stellate cirrhosis. The changes are most marked in the region of the larger portal spaces, but there is active proliferation of the connective tissue throughout Glisson's capsule, and every lobule is soon enclosed and encroached upon peripherally. The smaller necroses are replaced by proliferation of the neighboring liver cells, but with the larger

[9] Bürker, K., *Arch. ges. Physiol.*, 1900–01, lxxxiii, 241.

[10] Heidenhain, R., in Hermann, L., Handbuch der Physiologie, Leipsic, 1883, v, pt. 1, 256. Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[11] Richardson has an excellent brief description (Richardson, M. L., *J. Exp. Med.*, 1911, xiv, 401). For one more extensive, with a survey of the literature, see Fiessinger, N., and Roudowska, L., *Arch. méd. exp. et anat. path.*, 1914–15, xxvi,18.

this is impossible, owing to involvement of all the lobular elements, and organization takes place by a connective tissue invasion. The surviving parenchyma becomes more and more jaundiced, and numerous intralobular bile thrombi appear. Small scattered necroses continue to develop, but no large ones form after the first days. The lobules become very irregular and diminish in size through the encroachment of connective tissue, and the latter rapidly penetrates in thin strands between the cell cords to the central vein. Within 3 weeks the liver has become a roughly hobnailed organ, with ducts everywhere greatly distended, and a cirrhosis that is at once periportal, or, more properly speaking, pericholangitic, unilobular, irregularly intralobular where necroses have been replaced, and almost regularly intralobular here and there where the connective tissue penetration is diffuse. Throughout the older cirrhotic tissue are great numbers of new-formed bile ducts. Despite the extensive parenchymal destruction, the liver remains of about the normal size. Thrombosis of portal branches not infrequently occurs, with some resultant atrophy of liver parenchyma, while elsewhere an hypertrophy may be observed when the condition of the animal permits, as is rarely the case. Always there is great emaciation, and death usually ensues within 4 or 5 weeks. Jaundice appears after the first few days of obstruction.

No human lesion resembles this one more than remotely, and attempts to utilize it in explanation of the biliary cirrhoses of man have been almost given up. Yet when its histologic components are considered separately the fact becomes plain that certain of them find their replicas in one kind of human liver cirrhosis, and certain in another. In a word, the rabbit lesion is mixed, involving injury from stasis throughout the entire length of the biliary tract. By altering the conditions it is possible, as we shall show, to localize the injury of stasis to regions corresponding to ducts of a single level or order, and thus to obtain cirrhoses of uncomplicated type which closely resemble the biliary cirrhoses of man.

Results of Local Obstruction.

The results of tying off the bile duct to the main liver mass of the rabbit, some three-fourths of the entire organ, while leaving that to the lobe mass free have been briefly described by Nasse.[12] We have repeated his experiments and can confirm his statements as regards the early changes, these alone having importance for the present work.

[12] Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

The effects of the sudden stasis on the main liver, though outspoken, are far less marked than after total obstruction. Dilatation of the bile ducts and gall bladder is relatively moderate, biliary necroses are small and appear only during the first few days, and the animal remains in good condition, never becoming jaundiced. The occluded parenchyma shows no bile tinting after the 1st week. Cirrhosis makes its appearance gradually and differs from that of total stasis in its slow, orderly progress, almost complete failure to invade the lobuli, and, in the subordination of stellate growth about the larger ducts, to a diffuse interlobular proliferation (Fig. 1). But new-formed bile ducts are still a prominent feature. The parenchyma undergoes a rapid atrophy. Within a few weeks the mass is much smaller, diffusely cirrhotic, and relatively bloodless; and by the end of 4 months it is reduced to a tag from which parenchyma and new-formed bile ducts have alike disappeared.[12] There is only the slightest hobnailing at any time. As atrophy of the main liver progresses the lobe mass undergoes hypertrophy and eventually reaches the size of the entire normal organ.

The cirrhosis here approaches the pure unilobular in type, for penetration between the cell cords is almost wholly absent (Fig. 1). But there still exists the complication of a stellate periportal proliferation, with notable dilatation and pericholangitis of the larger ducts. Nasse attributed the atrophy to closure of the secretory channels of the liver, likening the result to that of ureteral ligation. This though, is no sufficient explanation, since under the conditions of total obstruction the liver holds its size, and there may even be some local hypertrophy despite the progressive general emaciation. With local obstruction similar attempts at repair are never seen, although the animal stays in good condition.

The Factor of Portal Obstruction.

It has seemed to us probable that the mildness of the biliary lesions, including the cirrhosis, after local obstruction, and the parenchymal atrophy as well, are due in great part to a single factor, namely, partial deflection of the portal stream from the affected region. Several facts support the view. When the duct from the main liver is tied the lobe mass becomes engorged with blood and hypertrophies just as when its portal blood supply is increased by obstructing the venous trunk to the main liver;[7] while a sufficient cause for obstruction is to be found in the dilatation under pressure of the main bile

channels of the liver. Betz showed in 1862[13] that a very moderate increase of pressure within the ducts, far less than that developing after total obstruction,[14] can greatly hamper the portal flow. That it does hamper it in the present case, and early, was indicated in one of our animals examined 5 days after ligation of the bile duct to the main liver. The lobe mass was already much engorged and enlarged, although as yet no connective tissue changes had occurred in the main liver that could be invoked to explain a venous obstruction there. The effect on hepatic tissue of the occlusion of portal branches has already been studied by us. There ensues an orderly atrophy with lessening of the biliary activity, and ultimately all parenchyma disappears. Precisely such an atrophy, though one more gradual, results from local biliary stasis; while a diminished biliary activity, at least as regards secretion into the ducts, is indicated by the moderate dilatation of the latter in comparison with their condition when stasis is total, and by the relative rarity of biliary necroses and the slow course of the connective tissue changes.

To obtain additional evidence we have studied the effects of obstructing simultaneously the bile duct and portal branch of a liver mass.

Experiment 1.—Two 1,500 gm. rabbits were operated upon under ether and the bile duct and portal trunk to the main liver twice ligated, just above the caudate lobe. The latter was not taken out. The livers appeared normal at operation, and subsequent examination of the hypertrophic lobe mass showed that cirrhosis had been absent. The animals were killed by bleeding from the heart 5 days and 21 days respectively after operation. They had been in good condition and unjaundiced.

In three other rabbits a mere ligation of the bile ducts to the lobe mass was followed by an occluding thrombosis of the accompanying portal branch. The occlusion must have developed soon after operation, for when the animals were killed the histological findings were identical with those observed in the main liver after simultaneous ligation of both duct and vein.

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[13] Betz, W., *Sitzungsber. k. Akad. Wissensch. Math.-naturw. Cl., Wien.*, 1862, xlvi, 238.

[14] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

Experimental Unilobular Cirrhosis.

Different stages of the same lesion were found in these rabbits. There was little dilatation of the large bile ducts and only a slight, transient jaundice of the tissue in stasis. A few small parenchymal necroses were seen early, but none later. The lobules rapidly diminished in size by simple atrophy, yet cirrhosis appeared more slowly than when the bile duct alone was obstructed, and, while ringing all the lobules about with a tissue containing many little, new-formed ducts, had not the least invasive tendency. It was, in sum, a pure, evenly distributed, monolobular cirrhosis. Stellate proliferation about the larger ducts was practically absent, as might have been expected from the absence of any marked stretching of their walls. With this exception the picture so entirely resembled that found after occlusion of a bile duct only (Figs. 1 and 2) as to constitute strong evidence for the view that obstruction of the portal stream has much to do with the lesions occurring under such circumstances.

Further Influence of the Portal Obstruction.

The deflection of the portal stream from a liver region in which the bile ducts are dilated as the result of obstruction is probably only partial at first. Later, when cirrhosis develops, it becomes much more nearly complete, as shown by the relatively bloodless condition of the tissue at a period when much parenchyma is still present. Perhaps the arterial circulation is now compromised. Certainly a portion of the blood that comes to the liver passes on to the vena cava through capillaries in the cirrhotic tissue[15] and thus fails of contact with the parenchyma. Pigment secretion into the ducts completely ceases; their dilatation, while still very moderate, comes to a standstill; and the fluid in them becomes colorless and watery. Yet, as our previous work has shown, a main liver mass long deprived of all portal blood, and in advanced atrophy as result, is still capable of forming bile in quantity.[7] The pressure, though, against which secretion takes place into the ducts is greatly diminished, as the following experiment proves.

[15] Ackermann, *Virchows Arch. path. Anat.*, 1880, lxxx, 396.

Experiment 2.—The portal trunk to the main liver mass was ligated in two rabbits of approximately 1,800 gm. weight, and the caudate lobe ablated as usual. 17 and 23 days later, respectively, the bile duct from the mass was ligated just below the entrance of the cystic duct, and 2 and 3 days later the animals were killed and immediately examined. The main liver had atrophied in both instances to less than one-third of the normal weight, while the posterior lobe mass had undergone a corresponding hypertrophy.

In a number of other rabbits the operative procedures were the same, but a gall bladder fistula was made in addition. Certain of the instances in which bile collection was successful have already been described.[7] Sometimes occlusion of the fistula developed, and the animals were killed at various periods thereafter. The findings in such cases deserve consideration with those of Experiment 2 proper, since the conditions are essentially similar.

In these animals with an advanced atrophy of the main liver as result of portal ligation, not the least icteric tinting of the parenchyma or dilatation of the bile passages and gall bladder followed occlusion of the duct; and the passages contained merely a little watery, colorless fluid. Obviously a very slight increase of pressure within the ducts had been sufficient to check secretion into the normal channels. It follows that as hepatic atrophy progresses after local biliary obstruction the chance that lesions will occur through rupture of the ducts, or stasis within them, becomes negligible. Yet when the material for bile formation is provided in unusual quantity a parenchyma deprived of the portal stream can secrete bile into the ligated duct against a considerable pressure, as will now be illustrated by an experiment done with another end in view.

Experiment 3.—A hemolytic and hemagglutinative serum of high titer was obtained by repeatedly injecting a goat with washed, rabbit red cells. A small amount of the serum when given intravenously to rabbits was found to bring about blood destruction over a period of many hours without demonstrable liver injury, whereas large quantities caused necroses. With a properly regulated dosage there was no escape of hemoglobin by way of the urine, even when more than half of the blood of the animal was broken down in the course of only 2 days.

The portal vein and bile duct of the main liver of a 1,700 gm. rabbit were ligated just below the entrance of the cystic duct. 4 days later, when the animal had recovered from the operation and was in good condition, intravenous injections were begun of small doses of the anti-rabbit serum, followed at short intervals by the transfusion of washed, compatible, rabbit red cells suspended in a little salt solution. The amount of serum given was always well below that causing liver changes, but it effected great blood destruction, necessitating five large transfu-

sions to maintain the hemoglobin at the original level. In a period of 11 days 73 cc. of sedimented corpuscles, an amount equivalent to the cell content of 162 cc. of the blood of the animal, or nearly twice the normal total, had been destroyed as the result of but four injections of serum, or 1.7 cc. in all. The animal now possessed a hemoglobin of 86.5 per cent (Palmer) as compared with 82.6 per cent prior to the experiment. It was killed and autopsied. There was at no time any jaundice.

Three rabbits were submitted to a similar experiment for a shorter period of time, and in two others repeated injections were made subcutaneously of large amounts of hemoglobin prepared by the method of Minot and Sellards. No jaundice was observed in any animal.

In these rabbits the bile passages of the main liver and the gall bladder as well were greatly distended and with an abnormally thick, dark green bile. The liver tissue was deeply jaundiced. The changes cannot be contrasted with those in the animals of Experiment 2, since atrophy of the main liver was advanced in the latter before the bile duct was obstructed. They should be compared with the results in Experiment 1, in which a bile duct and portal branch were simultaneously obstructed. Not only was the local accumulation of bile far more copious and the distension of the ducts greater than in the few instances of this experiment, but both were much more pronounced than in any of the rabbits in which, following Nasse's example, we ligated only the duct of the main liver, leaving the portal circulation untouched. It follows that the failure of a liver region deprived of the portal stream to secrete bile against pressure cannot be laid to an essential disability of the cells. In a previous paper evidence has been brought forward that the cells are handicapped in competition with the parenchyma receiving all the portal blood.[7]

Experimental Intralobular Cirrhosis.

A stellate cirrhosis about the larger bile ducts is not rare in human beings as the result of obstruction, with or without infection, and has frequently been brought about experimentally in animals. We have deemed its production in rabbits, apart from other changes, as unnecessary, although logically considered this would be a further step toward separating out the components of the mixed lesion that follows occlusion of the common duct. To obtain, on the other hand,

a type of stasis, and thus presumably of cirrhosis, localized within the lobules, seemed highly desirable. *A priori*, one would expect to meet many difficulties, since it is known that a back pressure throughout the duct system which suffices to cause partial stasis in the lobules almost invariably results in a rupture of some of the bile radicles within Glisson's capsule, and always in connective tissue proliferation about the larger ducts. Our success in obtaining a predominantly intralobular stasis has been the outcome of varied attempts. The condition, and its corollary, a diffuse intralobular cirrhosis, can regularly be brought about in the lobe mass of the liver by diverting the whole portal stream to the latter and ligating the efferent duct. A like result is not produced, we find, when the larger mass of the main liver is subjected to the same procedure, an experiment already performed by Steenhuis.[16] The cirrhosis that then occurs is predominantly extralobular.

Experiment 4.—In eighteen rabbits weighing from 1,500 to 2,400 gm. the bile duct of the posterior lobe—or ducts, for there may be as many as three—was ligated, the portal trunk to the main liver tied off just above the branch to the caudate lobe, and this last ablated as usual. The animals remained in good condition and unjaundiced. They were killed after intervals of from 1 to 33 days.

The operation as described is frequently followed by complications; and the findings in many animals not included in the above number have been ruled from consideration on this account.

The course of the changes has been carefully followed. There is an initial turgor and hypertrophy of the lobe mass resembling that after ablation of the main liver,[17] or simple ligation of the portal branch of the latter,[7] and due of course to the same marked, local increase in the portal stream. Superimposed are destructive changes referable to the bile stasis. The stasis, though brought about by a ligature on the principal duct of the lobe, causes remarkably little dilatation of the latter and still less of its large branches within Glisson's capsule. These are never abnormally prominent on the cut surface of the liver, though microscopically a slight distension and thickening of their walls may be observed after a time, with occasionally some pericholangitis. In the course of several weeks the main duct usually attains a diameter of about 3 mm., that is, becomes twice the normal size, and contains stasis bile, a watery fluid with a few green

[16] Steenhuis, T. S., Experimenteel en Kritisch Onderzoek over de Gevolgen van Poortaderafsluitung, Thesis, Gröningen, 1911.

[17] Ponfick, E., *Virchows Arch. path. Anat.*, 1889, cxviii, 209; 1890, cxix, 193; 1895, cxxxviii, suppl., 81.

solid particles. The brunt of the obstruction falls on the smallest bile radicles in Glisson's capsule, and still more on the canaliculi within the lobules themselves, as shown by the bile thrombi here found and the numerous, minute parenchymal necroses. Such necroses appear within a few hours after the ligation and new ones develop day by day. Usually they cannot be seen with the naked eye, involving, as they most often do, one to three or four cells scattered throughout the lobule from periphery to center. The dead cells stand forth prominently in the liver cords, staining a bright pink with eosin, and lacking nuclei (Fig. 3). By the 3rd day they are numerous, especially in the outer and mid-zone of the lobule, and occasionally a few larger necroses involving ten or fifteen cells may now be present. Some of the latter are undoubtedly the *Netz-Nekroses* which Steenhuis[16] saw in liver tissue hypertrophic as result of an increased portal flow, but others have the character of frank biliary necroses. Very exceptionally they involve considerable lobular segments and may then attract the unaided eye. In animals showing them the smaller, disseminated necroses are especially numerous. It is the latter that characterize the lesion and continue steadily to be formed. Being purely parenchymal they are rapidly replaced by proliferation of the lobular cords without connective tissue participation, and their number is never sufficient at any one time to render them confluent. They appear to have no relation to the small, scattered, intralobular bile thrombi that, during the first few days, and then only, are numerous enough to attract attention.

The severity of the lesion varies largely with the individual. By the 3rd day in some cases, or the 6th or 7th in others, a new element makes its appearance; namely, a proliferation in the finer ramifications of Glisson's capsule. Fibroblasts begin to penetrate within the enlarging lobules (Fig. 4), and by the 12th day as a rule many thin strands of them can be found, so far toward the central vein and so isolated in cross-section that were the absence of connective tissue within the normal parenchyma not well attested[18] one would conclude that they had developed *in situ*. New-formed bile ducts have now made their appearance in the interlobular connective tissue which is definitely increased in amount.

The lobe mass at this time is highly interesting in the gross. It is enlarged, sometimes to double its normal size, with rounded contours and blunt edges. The bulging hypertrophic lobules render the surface slightly and regularly uneven. The tissue is firm and somewhat inelastic, and the knife meets a smooth, non-crepitant resistance. The color is gray-pink, in contrast to the purple of the main liver. When the vessels are severed nearly all the blood is forced out by the turgid tissue, and the general tint is then a uniform, pale buff, or putty color. On the surface laid open the lobules can with difficulty be made out, slightly raised, in a matrix of gray, translucent, connective tissue which appears to penetrate and overgrow them. Sometimes their position can be made out only through the situation of the gaping central veins. There is now no definite jaundice of the tissue, though its yellowish tint is suggestive. Earlier, during the

[18] Mall, F. P., *Am. J. Anat.*, 1906, v, 227.

first few days after the ligation, the enlarged lobe may be a greenish red-purple, though jaundice of the animal never occurs.

By the 21st day the lobe mass, still in process of hypertrophy, contains nearly as much connective tissue as parenchyma. Almost everywhere fibroblasts have penetrated to the central veins of the lobules, though so regularly that the original pattern of the liver is still fairly maintained. The cell cords, though, are distorted and much fewer. The necrosis of individual cells and small cell groups is still going on, as is also proliferation of the surviving parenchymal elements which individually may be much above normal size. The interlobular connective tissue is greatly increased, both relatively and actually, and everywhere throughout it are new-formed bile ducts. About some of the small hepatic veins is a collar of young connective tissue containing in its midst irregular strands of small, compressed looking cells, superficially suggestive of new-formed bile ducts but in situation widely separate from the latter and with tinctorial differences that identify them as parenchymal.

At this time the lobe mass is of a pale flesh color and appears almost bloodless compared with the main liver (Fig. 9). In truth it is so, owing to interference with the portal stream by the cirrhotic tissue, which usually begins before the 12th day and has now become marked. Venous collaterals open, sometimes as a *caput Medusæ*, when the omentum is attached by adhesions to the old wound, more usually as small, direct anastomoses between the portal branches and vena cava, and oftenest as dilated Charpy's veins along the gastrohepatic omentum to the main liver. But all of the new channels fail as a rule to avert a serious chronic passive congestion. As result the spleen is changed into a tense cylinder (Fig. 9) and may weigh more than 4 gm., nearly six times the average for normal rabbits of similar size. Occasionally ascites occurs. On cutting into the lobe mass its large, portal channels are found to end bluntly in small veins.

The surface of the lobe is still finely rugose, and its substance is now extremely resistant to the knife, though non-crepitant. When laid open the glistening, bloodless, pinkish gray tissue shows no distinct pattern of lobules, but these can usually be made out when viewed obliquely, since they are slightly raised above the general surface. Some appear larger than normal whereas others blend with the connective tissue. The main liver mass fails to undergo the atrophy which in the lack of a functional need would be its fate.[7]

By the 30th day cirrhosis has progressed much further and so has the development of collaterals which shunt the portal blood around the lobe mass. The adequacy of the new channels is now shown by the disappearance of all signs of passive congestion. The lobe mass has in consequence come to lie outside of the venous stream, being reduced to much the same circulatory condition as if its portal branch had recently been tied. Hypertrophy ceases, and atrophy takes its place. But though the lobe mass is still much above normal size little parenchyma is left to undergo retrogression. The mass consists almost entirely of connective tissue with an abundance of new-formed bile ducts and some round cells. Here

and there are to be found small islands of liver cords, and separated cords and cells (Fig. 5), some of them dying, though whether as a result of the bile stasis or of the cirrhosis we have not sought to discover. The liver destruction has far surpassed that compatible with life were the whole organ affected. Beyond the 33rd day the changes have not been traced.

Altogether the type of cirrhosis is as purely intralobular as is compatible with the fact that the lobules normally contain no connective tissue,[18] so that its appearance and proliferation within them must of necessity take place by penetration from without.

Influence of an Increased Portal Stream.

The secretory activities of a lobe mass receiving the entire portal stream but with bile duct ligated, as in the preceding experiment, become strikingly evident when the duct from the main liver is also tied with result in total obstruction. There is now no relief for the lobe mass such as might be afforded through the efforts of a parenchyma with unobstructed bile outlet.

Experiment 5.—In ten rabbits of 1,500 to 2,200 gm. an operation similar to that of Experiment 4 was successfully performed and in addition the bile duct from the main liver mass was ligated. In eight cases the gall bladder was removed in order to render the conditions in the main liver more strictly comparable with those in the lobe mass. The animals died, or were killed, after periods of 1 to 12 days. Cultures taken from such as died showed that the liver lesions were not referable to infection; and as further proof the histological findings agreed in all ways with those of animals killed while still in good condition.

The animals of this experiment were more seriously affected than if total biliary stasis had been produced in the ordinary way by obstruction of the common duct. A sufficient explanation is to be found in the radical circulatory derangement.

Only two animals survived for as long as 12 days. Jaundice always developed within 48 hours, and rapid emaciation was the rule; yet despite the poor general condition the lobe mass of the liver underwent some hypertrophy. Its turgid tissue became brightly and evenly bile-stained—green, or ocher-colored. The surface remained smooth, and at no time were biliary necroses perceptible in the gross (Fig. 10). The lobules were definitely enlarged and appeared regular, save that near the central vein there was some opacity with a brilliant bile tinting. The impression here gained was not that of a diffuse necrosis but of intense im-

pregnation of living tissue with bile pigment. This was the actual state of affairs, as sections showed. Toward the center of the lobules pigment existed in great quantity as small bile thrombi (Eppinger), as large, rounded, brownish black masses (Fig. 6), intracellular particles, and as a diffuse stain for groups of dead parenchymal cells. No such marked condition has to our knowledge been encountered heretofore, though for that matter the factors in its production can scarcely be expected to come together naturally.

The earliest histological changes were like those observed in the companion Experiment 4, in which the duct of the main liver was left open. There was the same scattered necrosis of individual cells throughout the lobule, but in much more pronounced form (Fig. 7), and dilatation of the ducts was greater. Small concretion-like pigment "thrombi" appeared early here and there between the parenchymal cells, and after but a few days the pigmentary accretion was, as already described, enormous (Fig. 6). The number of dead cells grew rapidly, and close to the center of the lobules larger biliary necroses occurred, some deeply impregnated with bile, but none of such size as to attract the unaided eye. These features acted to modify the rapidly developing cirrhosis, which in the animals surviving longest was intralobular both by diffuse invasion and by the replacement of necroses. Pericholangitis of the larger ducts was absent. At the 12th day active division of the surviving parenchymal cells was still going on; some elements were of greatly increased size, and nuclear irregularities were frequent.

The main liver showed only the lesions that ordinarily follow obstruction of the common duct, combined with the slight atrophy inevitable to deprivation of the portal stream. The local accumulation of pigment was slight, as compared with that in the posterior lobe mass, yet large biliary infarcts were numerous (Fig. 8).

The extraordinary impregnation with pigment of the posterior lobe mass (Figs. 6 and 7) contrasts strikingly with the slight jaundice of the main liver (Figs. 8 and 10). Since biliary activity is dependent, in large part at least, upon portal flow, and this is greatly different in the two liver portions, such a contrast need not occasion surprise. The absence of gross necroses, though, in the posterior lobe mass is difficult to reconcile with the pronounced biliary leakage there occurring save on the assumption that the increased portal flow provided a circulation to cells that otherwise would have been deprived of it by the extravasations.

The Factor of Safety in Bile Elimination.

The tissue of the lobe mass in Experiment 4 was jaundiced only during the first few days after operation. Experiment 5 clearly shows that bile pigment must have been prevented from accumulating here,

as well as in the organism generally, through the activities of the main liver. When there exists only an uncomplicated biliary obstruction of the main liver, the posterior lobe mass functions in the same way to prevent a general jaundice, as has already been noted. It is remarkable that the literature contains no direct recognition of the fact that a small portion of the liver may serve for the whole as far as regards the elimination of bile pigment. On the contrary, one finds everywhere the statement that the severity of jaundice is directly proportional to the amount of liver tissue in stasis; and small, local lesions have been invoked to explain its clinical occurrence. The subject merits a separate paper and will be considered here only in so far as it affects our theme. But this it does to a considerable degree.

The factor of safety in bile elimination is strikingly illustrated by some recent experiments for another purpose performed by one of us and Dr. Philip D. McMaster. The bile duct from the main liver was tied, and so too was the portal branch to the lobe mass from which the caudate lobe was ablated as usual. Owing to the increased portal stream to the main liver the tissue of the latter must be supposed to have formed bile in unusual quantity, and this found no outlet by the normal route. Yet the eight rabbits of the series remained in excellent condition and unjaundiced, although the bile could be eliminated only through the lobe mass, less than a quarter of the total substance of the liver, and supplied with blood only through the hepatic artery, which, judging from observations on dogs,[19] provided only about 40 per cent of the normal blood supply.

Local Effects of Increased Bile Secretion.

It has repeatedly been suggested on the basis of clinical evidence that increased secretion, or the secretion of an abnormally thick bile, may lead to stasis within the ducts and thus injure the liver eventually. Hanot's cirrhosis was at one time attributed to such cause. But certainly small portions of the liver of the rabbit can rid the body of large amounts of the animal's highly irritant bile without suffering injury. This was true in all of the instances of compensatory secre-

[19] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, xxxv, 87.

tion described in the present paper. Even when the bile is thickened as the result of hemolysis and the whole brunt of its elimination is abruptly thrown upon the lobe mass of the liver, the latter suffers no damage visible histologically. Experiment 3 was originally devised to test this point.

DISCUSSION.

Physiological Pathology of the Lesions.

Rabbit bile that has escaped from the ducts is highly injurious to the tissues, causing necrosis even of the liver parenchyma.[20] In this fact and in the differing paths by which the fluid may leave the collecting tract under various conditions of obstruction are to be found primary reasons for the diverse types of biliary cirrhosis which we have produced experimentally.

The cirrhosis which follows ligation of the common duct is, as has already been pointed out, a mixed lesion. So considerable is bile secretion despite the stasis, that no part of the collecting tract from the intralobular canaliculi to the main duct, inclusive, escapes the effects of the irritant fluid. Pressure changes also occur, and the local condition is probably aggravated by the resecretion of biliary constituents from the heavily charged blood,[21] which would greatly favor accumulation within the hepatic tissue. Though most of the secretion must continue to find its way back into the blood and lymph, the process cannot but be faulty when the body fluids are already heavily laden.

The progressive atrophy and orderly cirrhosis that result from obstruction of a single large bile duct are traceable to the interaction of two opposing sets of factors, one set vicious, the other tending to relieve the conditions. The initial bile stasis leads to rupture of some of the finer ducts with parenchymal necrosis, and its continuance to an interlobular and pericholangitic connective tissue growth. Probably this cirrhosis interferes eventually with the general blood

[20] Bunting, C. H., and Brown, W. H., *J. Exp. Med.*, 1911, xiv, 445.

[21] Bile constituents injected into the circulation are rapidly taken out by the normal liver—*vide* Stadelmann, E., Der Icterus, und seine verschiedenen Formen Stuttgart, 1891.

supply. There is no doubt that the portal flow is partially diverted from the beginning through the encroachment of distended bile ducts on the stream bed, and hence there ensues a gradual atrophy of the parenchyma such as follows any local portal deprivation.[7] But while destruction is thus being effected through a combination of several influences others act to limit the injury. The liver portion with duct still open keeps the organism more or less free from bile and thus tends to prevent resecretion into the region of stasis; secretion is still further reduced by the diverted portal flow;[7] and the direct effects of such stasis as nevertheless ensues are lessened by the passage of biliary constituents from the obstructed tissue into a blood and lymph relatively free from them. As parenchymal atrophy advances the pressure under which bile is secreted into the ducts decreases greatly (Experiment 2), and in consequence their rupture, with extravasation, no longer occurs. Furthermore, with the passing of time the functional responsibilities of the tissue in stasis decrease, owing to hypertrophy of the unobstructed liver mass. And so through several means the local changes are rendered mild and orderly. Similar lesions from similar causes, but with less of cirrhosis and a more rapid atrophy, as would naturally follow from the circumstances of the case, are seen when a bile duct and its corresponding portal branch are occluded at the same time (Experiment 1).

The predominantly monolobular cirrhosis which follows the occlusion of a single duct, and the purely monolobular lesion that develops when the corresponding portal branch is also tied, are referable not to rupture of the ducts, which is negligible, but to continued passage through their walls of the irritant bile. When a colored solution is introduced into the bile passages under a pressure that fails to rupture them it escapes with ease through the walls of the finest radicles in Glisson's capsule.[18] And precisely here, as indicated by the connective tissue lesion, does the bile pass into the tissues under the circumstances we are now discussing. This path of egress would seem sufficient to relieve the stasis when secretion has been cut down by the ligation of the portal branch to the affected region, for under such circumstances lesions are not observed elsewhere in the liver. When the duct alone has been ligated secretion and stasis are both greater, and in addition to the monolobular cirrhosis a stellate proliferation is then found about the large, distended ducts.

No clear reason is evident for the intralobular situation of the stasis that results from obstructing a single bile duct and greatly increasing the local portal flow. The changes that ensue, though, are easily understood. An increased portal flow of itself fails to induce connective tissue changes.[17] They may be attributed in the present instance to the numerous bile leaks within the lobules, leaks which also cause a profusion of punctate necroses. The lymph from the affected tissue comes away more or less laden with bile. It passes, not direct from the lobules into the formed lymphatics of Glisson's capsule, but first through unlined spaces between the connective tissue cells.[18] Here its biliary component has abundant opportunity to cause irritation, and here consequently at the edge of the lobules connective tissue proliferation occurs, and fibroblasts penetrate rapidly between the liver cords, following up, so to speak, the path of the irritant fluid. A diffusely intralobular cirrhosis is the result. The absence of large biliary infarcts, despite the great secretory activity of the tissue (Experiment 5), may be due in part to the support given the walls of the bile radicles by the increased portal pressure, which would also act to maintain the blood current despite local extravasations so that the latter affect relatively few cells. The observation has been made (Experiment 5) that when there is an increased portal flow in one part of the liver, an absence of such flow in the remainder, and biliary obstruction in both, gross biliary necroses fail to occur in the first mentioned liver portion, although the jaundice there is extreme, whereas in the second portion, which is unjaundiced, and must be relatively inactive, large infarct-like necroses appear (Fig. 8). To judge from this there would appear to be much in the current belief that biliary necroses, though caused in some part by the direct injury of cells by extravasated bile, owe their size when large to interruption of the blood current.

Interpretation of Human Lesions.

Many facts warrant the application of the principles here set forth to the frank biliary lesions of human beings. The essential likeness of the changes in man and the rabbit has been briefly indicated in an earlier part of this paper. As Quincke and Hoppe-Seyler put the

matter, summing up their more extensive comparison: "From all this it would seem that the findings in guinea pigs and rabbits are analogous in many ways to those in human beings."[5] But it is in essentials only that the findings are analogous. The relatively innocuous character of human bile, the slow rate at which it is secreted, the greater frequency of infection in areas of stasis, all act to vary the picture. Furthermore, the liver parenchyma may, like that of the dog, respond rather slowly to changed circulatory conditions[7] such as bile stasis induces. Certainly the intralobular bile canaliculi are, like those of the dog and cat, relatively inaccessible to pressure exerted through the large ducts,[18] so that they are only slightly implicated by stasis in these latter.

The forms of cirrhosis associated with Hanot's name cannot on present evidence be classed as of frank biliary origin. Nevertheless, our observations bring something of clarity into a consideration of them. Whether there exists an entity of the sort described by Hanot has been much debated, and there is great discordance in its definition by recognized authorities. No one disputes, on the other hand, the occurrence in man of a peculiar, progressive, hepatic cirrhosis, accompanied by more or less jaundice, and resulting usually in an enlarged liver in which are found inflammatory lesions of the smaller bile ducts with signs of stasis in them. It is our belief that the diversity of the liver changes is referable to differences in the duct levels at which the injurious agent is active, differences which in the rabbit lead, as we have shown, to cirrhoses of strikingly individual character. In rabbits, and with bile as the irritant, a localization to the intralobular bile canaliculi leads to a diffuse intralobular cirrhosis, whereas when the smallest bile radicles in Glisson's capsule are affected there results a pure monolobular cirrhosis, and implication of the large bile channels leads to a stellate proliferation about them. Parallel instances, pure and mixed, of all except the last mentioned lesion, are to be found within the group of the hypertrophic biliary cirrhoses of man. The rapidly progressive type of the disease, frequent in Indian children, is almost entirely of intralobular character, as Gibbons' excellent description shows.[22] There

[22] Gibbons, J. B., *Sc. Mem. Med. Off. India*, 1891, vi, 51.

is here a marked degeneration of the liver cells. According to Kaufmann,[23] with whom relatively few agree, intralobular growth is a distinctive character of Hanot's disease. In Lereboullet's monograph[24] most of the instances are monolobular, with occasionally an intralobular and sometimes a perilobular involvement.

The hypertrophy of the surviving parenchyma in Hanot's cirrhosis is not more difficult to understand than that usual to atrophic cirrhosis and here regenerative in character.[25] In explanation of the continued connective tissue growth which renders the liver large, one need only invoke the constant stimulus to proliferation that is undoubtedly present, as the angiocholitic lesions show. By contrast, injury is intermittent in the atrophic cirrhosis of Laennec, and at death, when a contracted scarring is found, often no signs of recent damage can be made out. The round cell aggregations present in hypertrophic biliary cirrhosis need no other cause than the chronicity of the inflammation. And the not infrequent chronic passive congestion occurring at a late stage with or without a marked diminution of the size of the liver, is referable to secondary connective tissue shrinkage and to the localization of the cirrhosis in the individual case. In our rabbits with hypertrophic biliary cirrhosis chronic passive congestion regularly developed as a late feature.

The ability of the least harmful bile of which the effects have been carefully followed, that of the cat, to elicit eventually a liver cirrhosis in the absence of infection[6] might well lead one to stress this factor as a cause of connective tissue changes in the human liver. The occurrence of intense local bile-staining without such lesions, a phenomenon not infrequent at autopsy in liver diseases, carries no weight in this connection, since the chronicity of the local stasis is here unattested. But Hanot's cirrhosis has certainly a varied etiology. The infantile form so frequent in India probably has its own specific cause. A number of well known microorganisms have been isolated from the liver in occidental forms of the disease, though the readiness with which infection supervenes on bile stagnation leads one to doubt

[23] Kaufmann, E., Lehrbuch der speziellen pathologischen Anatomie, Berlin, 1907.

[24] Lereboullet, P., Les cirrhoses biliaires, Thèse de Paris, 1902.

[25] Kretz, R., *Wien. klin. Woch.*, 1900, xiii, 271.

that their rôle is primary. Hanot's early hypothesis of a primary bile stagnation as the result of a "biliary diathesis" seems to us worthy of reconsideration in certain cases. The essential chronicity of the changes accords well with the relatively innocuous character of human bile; while the enlargement of the spleen which is sometimes great, and the occasional familial character of the malady are facts suggesting an inherent peculiarity of the patient closely akin to that of congenital familial jaundice, as several authors have not failed to point out. Yet it should be remarked in this connection that cirrhosis is not a feature of the latter disease, though for years the liver secretes a tenacious, heavily pigmented bile. Furthermore, we have been unable to cause liver injury in the rabbit by many times increasing the bile output of pigment from small portions of parenchyma (Experiment 3).

The possibility should be borne in mind that local bile stasis may act to complicate any chronic liver derangement in which bile passages, small or large, are compromised. That obstruction to the smaller channels may be responsible for certain of the supposedly "unobstructive" jaundices has been proved by Eppinger. Yet the absence of clinical jaundice is, as we have found, compatible with a condition of total obstruction in more than three-fourths of the liver of the rabbit. Should the same hold true for the human liver the existence of a "*cirrhosis biliaire anicterique*" such as has been described by French authors will not be difficult to understand.

SUMMARY.

There are excellent reasons for employing the rabbit in an experimental analysis of the biliary factor in liver lesions; and it is possible to obtain in this animal results uncomplicated by infection or by intercurrent cirrhosis.

Ligation of the common duct of the rabbit results in a mixed lesion from injury throughout the entire length of the bile channels. By obstructing single ducts and altering the portal stream we have produced cirrhoses of pure monolobular and diffusely intralobular types. The character of the connective tissue changes is determined by the path of escape of bile from the collecting system, which in turn is

largely conditional upon the secretory activity, while this again is dependent upon blood flow. The portal flow is largely diverted from regions of local stasis through encroachment on the stream bed by the dilated ducts.

There is a large margin of safety in bile elimination by the normal hepatic tissue. Less than a quarter of the liver of the rabbit, and this deprived of its entire portal stream, will suffice to keep the organism free from clinical jaundice and healthy when the remainder of the liver, which receives all of the portal blood, has its ducts ligated. The vicarious elimination thus illustrated is of great importance for regions of local stasis by keeping the blood relatively free from bile, thus preventing resecretion into such regions and facilitating exchange from them into the body fluids.

Our experimental monolobular and intralobular cirrhoses are the result of the limitation of biliary lesions to special levels of the duct system. Their resemblance to the different forms of "biliary" cirrhosis associated with Hanot's name is close, and the diverse liver lesions of Hanot's disease are readily explained on the assumption that the stasis, with or without infection, which is indubitably here present, has its situation at different levels in different cases. There are reasons for the view that bile stasis *per se* may sometimes be a prime cause of the malady. Certainly such stasis must be thought of as acting to complicate many chronic liver lesions.

In a later paper experiments on the dog will be described essentially similar in result to those on the rabbit as here set forth.

EXPLANATION OF PLATES.

PLATE 17.

FIGS. 1 and 2. The lesions after 14 days occlusion of the bile duct from the main liver mass (Fig. 1), and after 22 days of such occlusion plus ligation of the corresponding portal trunk (Fig. 2). There is an identical interlobular cirrhosis in both cases with many new-formed bile ducts, simple atrophy of the parenchyma, and a dwindling in size of the lobules. The scattered dark spots are pigmented Kupffer cells such as are found after any local portal obstruction. Hematoxylin and eosin.

PLATE 18.

FIG. 3. Condition of the lobe mass of the liver 2 days after the operation to produce hypertrophic cirrhosis. Necrotic parenchymal cells can be seen here and there. Two are indicated by arrows. The lacunæ near the center of the

lobule on the left indicate where bile thrombi have been dissolved out by the fixative. Eosin and methylene blue.

FIG. 4. Experimental hypertrophic cirrhosis after 13 days. In the center of the field is an unusually well preserved lobule with fibroblasts penetrating it from all sides. Elsewhere only irregular cell cords can be distinguished amid the cirrhotic tissue. Hematoxylin and Van Gieson's stain.

PLATE 19.

FIG. 5. Experimental hypertrophic cirrhosis after 30 days. The parenchyma is almost entirely replaced by connective tissue containing many new-formed bile ducts. The position of the lobules can no longer be made out save from the central veins. One of the latter is here shown with some degenerating liver cords near it, while elsewhere a few parenchymal cells can be seen. Eosin and methylene blue.

FIG. 6. Pigment accumulation in the lobe mass 7 days after diversion to it of the entire portal stream and ligation of the common duct. Masses of bilirubin are to be seen, especially near the center of the lobules. There is a beginning cirrhosis. Fresh specimen stained with methylene blue.

PLATE 20.

FIGS. 7 and 8. The lesions 7 days after diversion of the whole portal stream to the lobe mass and ligation of the common duct. In Fig. 7, of the lobe mass, one finds a punctate parenchymal necrosis which is far more pronounced than when biliary obstruction is local (Fig. 3), while the number of bile thrombi, as indicated by lacunæ, is much greater. In the main liver (Fig. 8) necrosis takes a gross form although the other signs of stasis are slight. Both specimens have the same magnification. The cells of the lobe mass are relatively very large. Eosin and methylene blue.

PLATE 21.

FIG. 9. Experimental hypertrophic cirrhosis after 21 days. The fleshy, pink mass with slightly roughened surface next to the pylorus is the cirrhotic lobe mass, well nigh bloodless owing to the obstruction of its vessels. The spleen, in chronic passive congestion, is a much enlarged, tense cylinder, despite the collateral vein running from it to the main liver. There was marked ascites in this instance.

PLATE 22.

FIG. 10. Results of 7 days diversion of the portal stream to the lobe mass of the liver with ligation of the common duct. The lobe mass is enlarged and deeply jaundiced, but without evident necroses, whereas the main liver, somewhat smaller than normal and almost unjaundiced, shows large biliary infarcts.

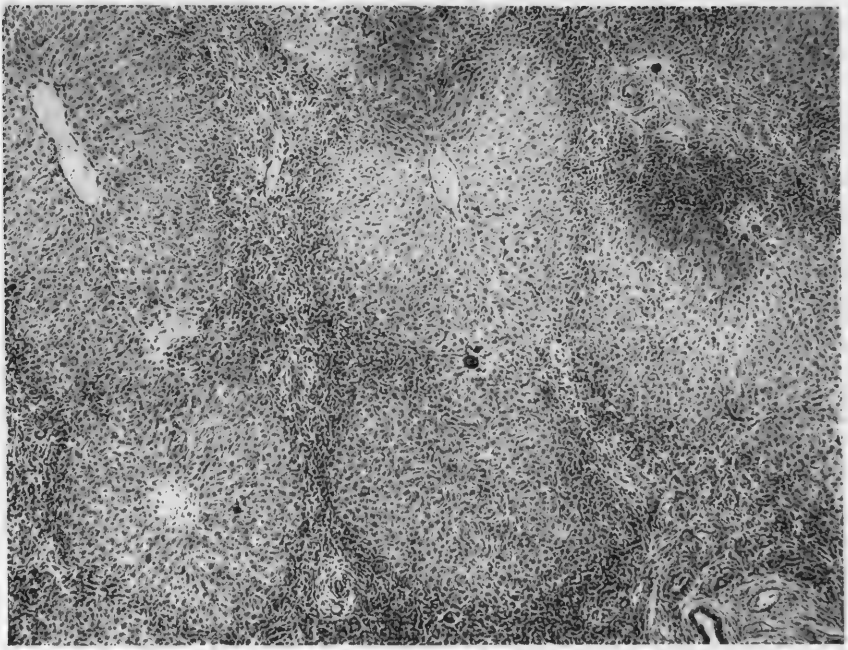

FIG. 1.

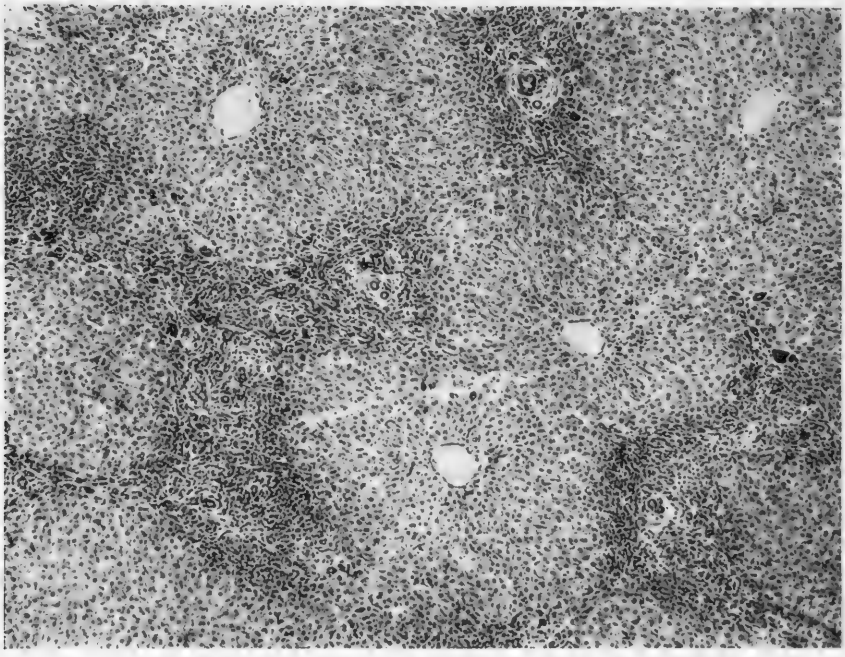

FIG. 2.

(Rous and Larimore: The biliary factor in liver lesions.)

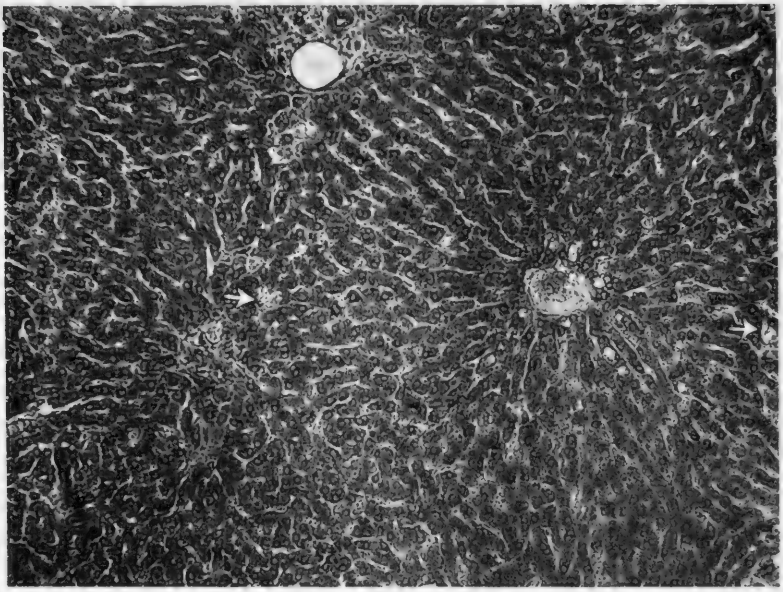

FIG. 3.

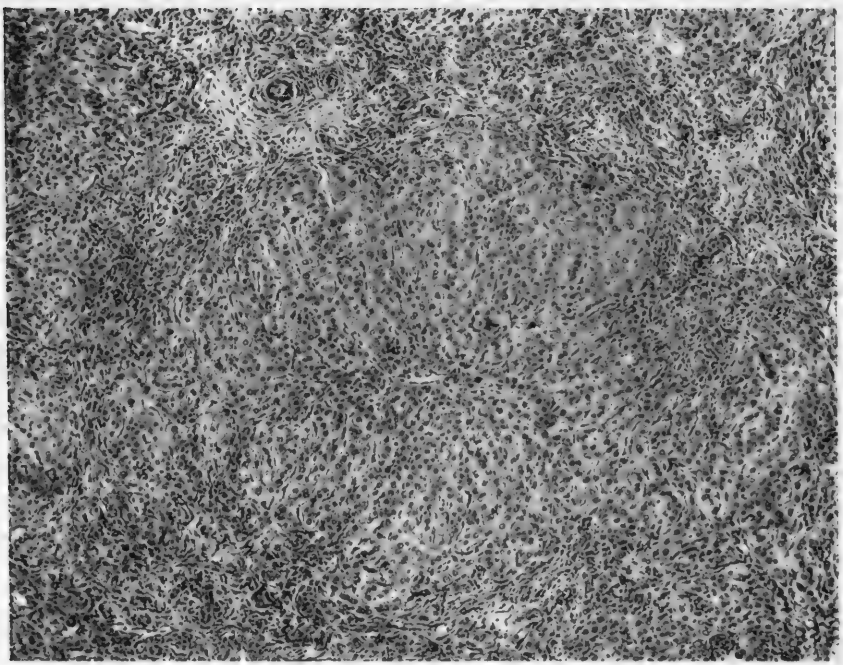

FIG. 4.

(Rous and Larimore: The biliary factor in liver lesions.)

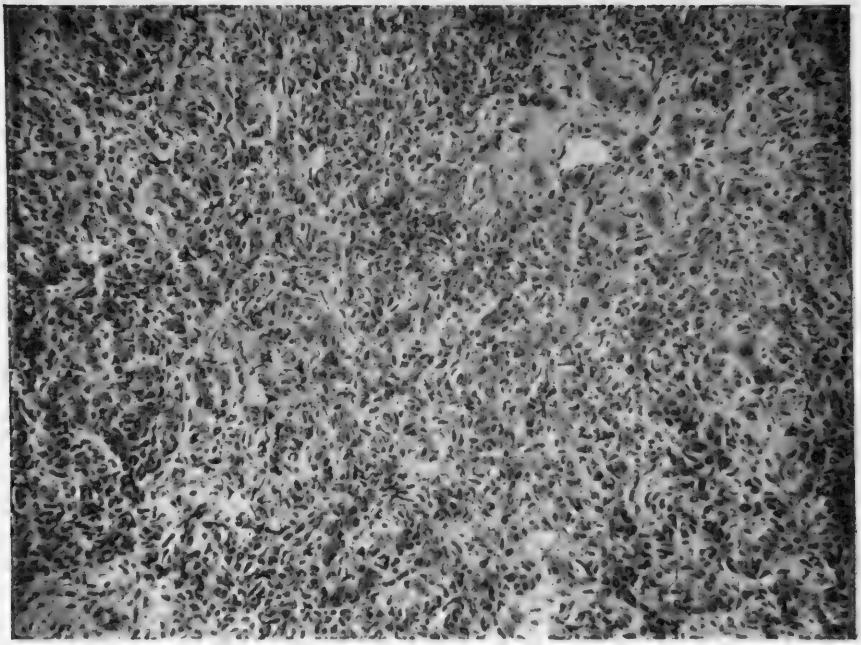

FIG. 5.

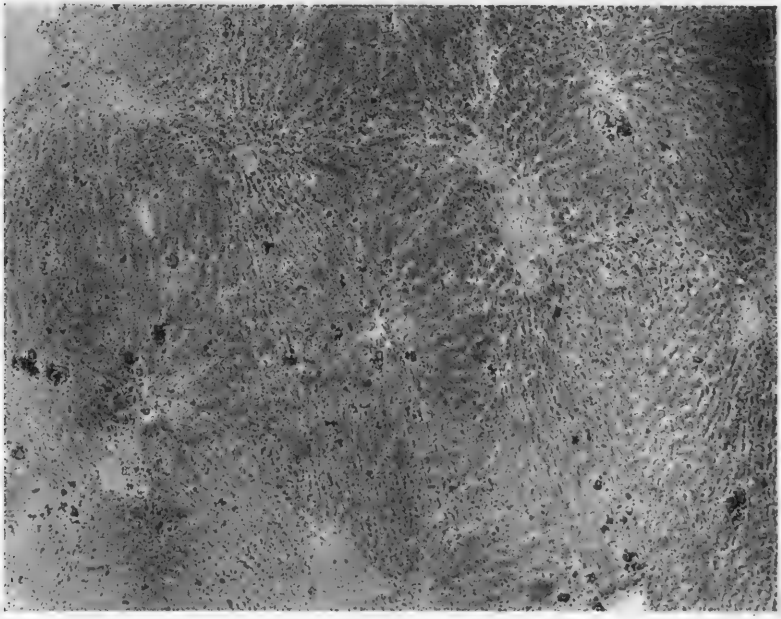

FIG. 6.

(Rous and Larimore: The biliary factor in liver lesions.)

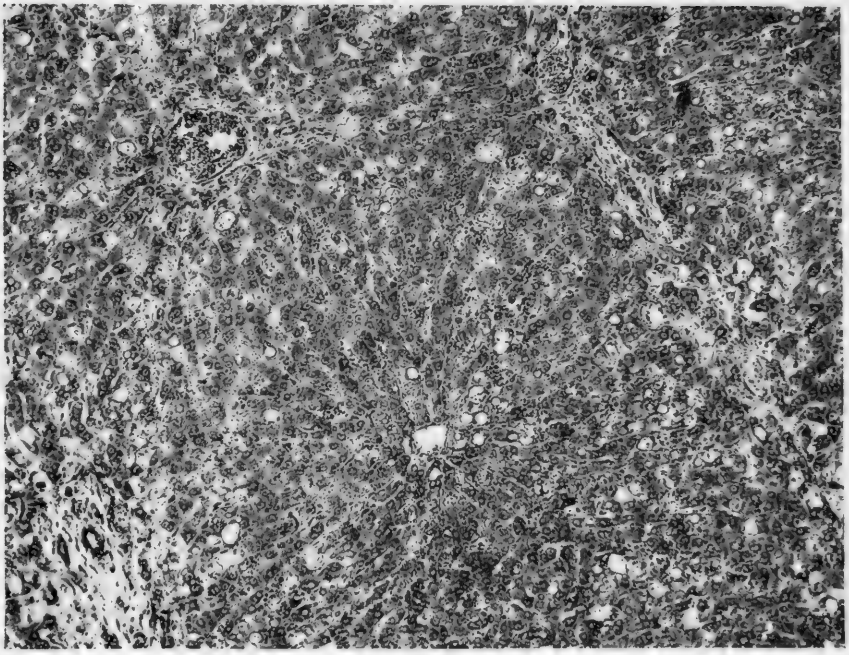

FIG. 7.

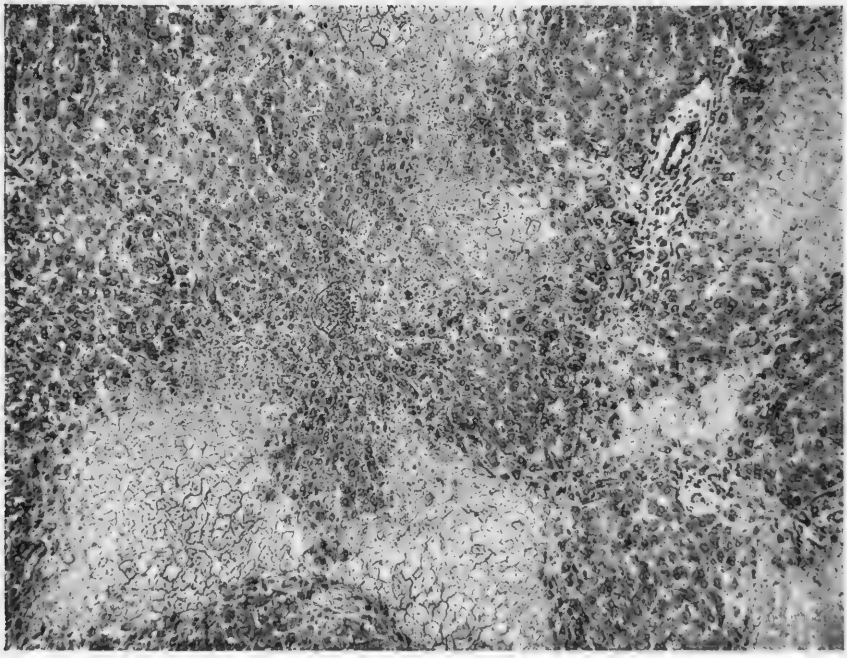

FIG. 8.

(Rous and Larimore: The biliary factor in liver lesions.)

FIG. 9.

(Rous and Larimore: The biliary factor in liver lesions.)

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FIG. 10.

(Rous and Larimore: The biliary factor in liver lesions.)

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